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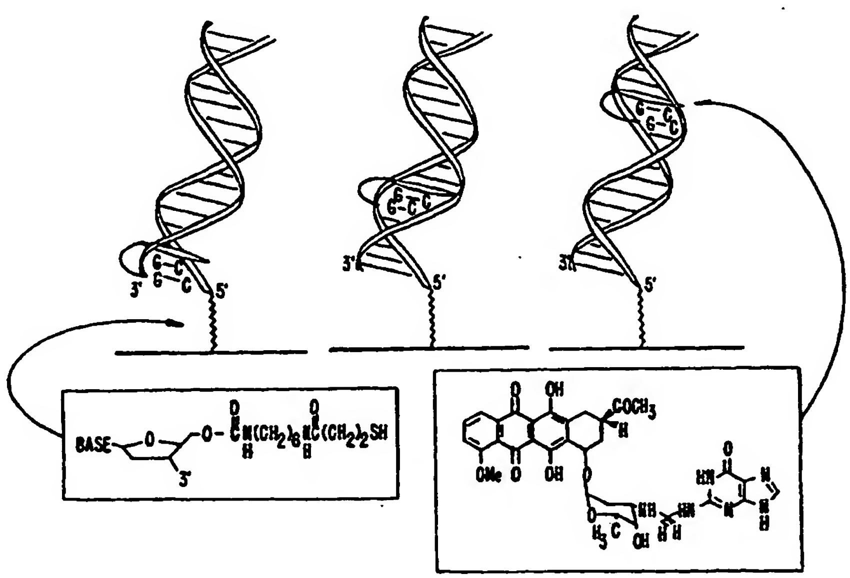

(57) Abstract

Compositions and methods for electrochemical detection and localization of genetic point mutations and other base-stacking perturbations within oligonucleotide duplexes adsorbed onto electrodes and their use in biosensing technologies are described. An intercalative, redox-active moiety (such as an intercalator or nucleic acid-binding protein) is adhered and/or cross-linked to immobilized DNA duplexes at different separations from an electrode and probed electrochemically in the presence or absence of a non-intercalative, redox-active moiety. Interruptions in DNA-mediated electron-transfer caused by base-stacking perturbations, such as mutations or binding of a protein to its recognition site, are reflected in a difference in electrical current, charge and/or potential.

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ELECTROCHEMICAL SENSOR USING INTERCALATIVE, REDOX-ACTIVE MOIETIES

1. RELATED APPLICATION

This application is a continuation-in part of Serial No. 60/043,146, filed April 9, 1997.

2. GOVERNMENT RIGHTS

The U.S. Government has certain rights in this invention pursuant to Grant No. GM 49216 awarded by the National Institute of Health.

3. FIELD OF THE INVENTION

The present invention relates to the detection and localization of base-pair mismatches and other perturbations in base-stacking within an oligonucleotide duplex.

4. DESCRIPTION OF RELATED ART

It is now well known that mutations in DNA can lead to severe consequences in metabolic functions (e.g., regulation of gene expression, modulation of protein production) which ultimately are expressed in a variety of diseases. For example, a significant number of human cancers are characterized by a single base mutation in one of the three ras genes (Bos, 1989). In order to unravel the genetic components of such diseases, it is of utmost importance to develop DNA sensors that are capable of detecting single-base mismatches rapidly and efficiently and to establish routine screening of disease-related genetic mutations based on such sensors (Skogerboe, 1993; Southern, 1996; Chee, 1996; Eng, 1997).

Various methods that have been developed for the detection of differences between DNA sequences rely on hybridization events to differentiate native versus mutated sequences and are limited by the small differences in base-pairing energies caused by point mutations within extended polynucleotides (Millan, 1993; Hashimoto, 1994; Xu, 1995; Wang, 1996; Lockhart, 1996; Alivisatos, 1996; Korriyoussoufi, 1997; Elghanian, 1997; Lin, 1997; Herne, 1997). Typically, a nucleic acid hybridization assay to determine the presence of a particular nucleotide sequence (i.e. the "target sequence") in either RNA or DNA comprises a multitude of steps. First, an oligonucleotide probe having a nucleotide sequence complementary to at

least a portion of the target sequence is labeled with a readily detectable atom or group. When the labeled probe is exposed to a test sample suspected of containing the target nucleotide sequence, under hybridizing conditions, the target will hybridize with the probe. The presence of the target sequence in the sample can be determined qualitatively or quantitatively in a variety of ways, usually by separating the hybridized and non-hybridized probe, and then determining the amount of labeled probe which is hybridized, either by determining the presence of label in probe hybrids or by determining the quantity of label in the non-hybridized probes. Suitable labels may provide signals detectable by luminescence, radioactivity, colorimetry, x-ray diffraction or absorption, magnetism or enzymatic activity, and may include, for example, fluorophores, chromophores, radioactive isotopes, enzymes, and ligands having specific binding partners. However, the specific labeling method chosen depends on a multitude of factors, such as ease of attachment of the label, its sensitivity and stability over time, rapid and easy detection and quantification, as well as cost and safety issues. Thus, despite the abundance of labeling techniques, the usefulness, versatility and diagnostic value of a particular system for detecting a material of interest is often limited.

Some of the currently used methods of mismatch detection include single-strand conformation polymorphism (SSCP) (Thigpen, 1992; Orita, 1989), denaturing gradient gel electrophoresis (DGGE) (Finke, 1996; Wartell, 1990; Sheffield, 1989), RNase protection assays (Peltonen and Pulkkinen, 1986; Osborne, 1991), allele-specific oligonucleotides (Wu, 1989), allele-specific PCR (Finke, 1996), and the use of proteins which recognize nucleotide mismatches, such as the E. coli mutS protein (Modrich, 1991).

In the first three methods, the appearance of a new electrophoretic band is observed by polyacrylamide gel electrophoresis. SSCP detects the differences in speed of migration of single-stranded DNA sequences in polacrylamide gel electrophoresis under different conditions such as changes in pH, temperature, etc. A variation in the nucleotide base sequence of single-stranded DNA segments (due to mutation or polymorphism) may lead to a difference in spatial arrangement and thus in mobility. DGGE exploits differences in the stability of DNA segments in the

presence or absence of a mutation. Introduction of a mutation into double-stranded sequences creates a mismatch at the mutated site that destabilizes the DNA duplex. Using a gel with an increasing gradient of formamide (denaturation gradient gel), the mutant and wild-type DNA can be differentiated by their altered migration distances. The basis for the RNase protection assay is that the RNase A enzyme cleaves mRNA that is not fully hybridized with its complementary strand, whereas a completely hybridized duplex is protected from RNase A digestion. The presence of a mismatch results in incomplete hybridization and thus cleavage by RNase A at the mutation site. Formation of these smaller fragments upon cleavage can be detected by polyacrylamide gel electrophoresis. Techniques based on mismatch detection are generally being used to detect point mutations in a gene or its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of tumor samples. In addition to the RNase A protection assay, there are other DNA probes that can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Smooker and Cotton, 1993; Cotton, 1988; Shenk, 1975. Other enzymatic methods include for example the use of DNA ligase which covalently joins two adjacent oligonucleotides which are hybridized on a complementary target nucleic acid, see, for example Landegren (1988). The mismatch must occur at the site of ligation.

Alternatively, mismatches can also be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes (Cariello, 1988). With either riboprobes or DNA probes, the cellular mRNA or DNA which may contain a mutation can be amplified using polymerase chain reaction (PCR) prior to hybridization. Changes in DNA of the gene itself can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

DNA sequences of the specified gene which have been amplified by use of PCR may also be screened using allele-specific oligonucleotide probes. These probes are nucleic acid oligomers, each of which is complementary to a corresponding segment of the investigated gene and may or may not contain a known mutation. The assay is performed by detecting the presence or absence of a hybridization signal for

the specific sequence. In the case of allele-specific PCR, the PCR technique uses unique primers which selectively hybridize at their 3'-ends to a particular mutated sequence. If the particular mutation is not present, no amplification product is observed.

In addition, restriction fragment length polymorphism (RFLP) probes for the gene or surrounding marker genes can be used to score alteration of an allele or an insertion in a polymorphic fragment. However, since the recognition site of restriction endonucleases ranges in general between 4 to 10 base pairs, only a small portion of the genome is monitored by any one enzyme.

Another means for identifying base substitution is direct sequencing of a nucleic acid fragment. The traditional methods are based on preparing a mixture of randomly-terminated, differentially labeled DNA fragments by degradation at specific nucleotides, or by dideoxy chain termination of replicating strands (Maxam & Gilbert, 1980; Sanger, 1977). Resulting DNA fragments in the range of 1 to 500 basepairs are then separated on a gel to produce a ladder of bands wherein the adjacent samples differ in length by one nucleotide. The other method for sequencing nucleic acids is sequencing by hybridization (SBH, Drmanac, 1993). Using mismatch discriminative hybridization of short n-nucleotide oligomers (n-mers), lists of constituent n-mers may be determined for target DNA. The DNA sequence for the target DNA may be assembled by uniquely overlapping scored oligonucleotides. Yet another approach relies on hybridization to high-density arrays of oligonucleotides to determine genetic variation. Using a two-color labeling scheme simultaneous comparison of a polymorphic target to a reference DNA or RNA can be achieved (Lipshutz, 1995; Chee, 1996; Hacia, 1996).

Each of these known prior art methods for detecting base pair mismatches has limitations that affect adequate sensitivity, specificity and ease of automation of the assay. In particular, these methods are unable to detect mismatches independent of sequence composition and require carefully controlled conditions, and most methods detect multiple mismatches only. Additional shortcomings that limit these methods include high background signal, poor enzyme specificity, and/or contamination.

Over the last decade, attention has also focused on DNA as a medium of charge transfer in photoinduced electron transfer reactions and its role in mutagenesis and carcinogenesis. For example, studies were performed using various octahedral metal complexes (which bind tightly to DNA by intercalation) as donors and acceptors for photoinduced electron transfer. Dppz complexes of ruthenium, osmium, cobalt, nickel, and rhenium showed tight intercalative binding and unique photophysical and electrochemical properties. No photoluminesence was observed upon irradiation of the metal complexes in aqueous solution in absence of DNA (as a result of quenching by proton transfer from the solvent), whereas in the presence of DNA excitation of the complex afforded significant, long-wavelength emission (because now the intercalated complex was protected from quenching). Studies using rhodium intercalators containing phenanthrenequinone-diimine (phi) ligands displayed tight DNA binding by preferential intercalation, some with affinities and specifities approaching DNA-binding proteins.

Photoinduced electron transfer using DNA as a molecular bridge has been established in various systems. Using metal complexes intercalated into the base stack of DNA as donor and acceptor it has been proposed that the DNA π-stack could promote electron transfer at long range. Additionally, the products of redox-triggered reactions of DNA bases have been detected at sites remote from intercalating oxidants (Hall, 1996; Dandliker, 1997; Hall, 1997; Arkin, 1997). For example, it has been shown that a metallointercalator can promote oxidative DNA damage through long-range hole migration from a remote site. Oligomeric DNA duplexes were prepared with a rhodium intercalator covalently attached to one end and separated spatially from 5'-GG-3' doublet sites of oxidation. Rhodium-induced photooxidation occurred specifically at the 5'-G in the 5'-GG-3' doublets and was observed up to 37 Å away from the site of rhodium intercalation. In addition it was found that rhodium intercalators excited with 400 nm light, initiated the repair of a thymine dimer incorporated site-specifically in the center of a synthetic 16-mer oligonucleotide duplex. The repair mechanism was thought to proceed *via* oxidation of the dimer by the intraligand excited state of the rhodium complex, in which an electron deficiency (hole) is localized on the intercalated phi ligand. Like electron transfer between

metallointercalators, the efficiencies of long-range oxidative processes were found to be remarkably sensitive to the coupling of the reactants into the base stack (Holmlin, 1997) and depended upon the integrity of the base stack itself (Kelley, 1997c, 1997d; Hall, 1997; Arkin, 1997) as well as on the oxidation potential. Perturbations caused by mismatches or bulges greatly diminished the yields of DNA-mediated charge transport.

Other studies have reported electron transfer through DNA using nonintercalating ruthenium complexes coordinated directly to amino-modified sugars at the terminal position of oligonucleotides (Meade, 1995). In this system it was suggested that electron transfer is protein-like. In proteins, where the energetic differences in coupling depend upon σ-bonded interactions, small energetic differences between systems do not cause large differences in electronic coupling. In the DNA double helix however, π-stacking can contribute to electronic coupling such that small energetic differences could lead to large differences in coupling efficiency. Most recently, Lewis and coworkers measured rates of photo-oxidation of a guanine base in a DNA hairpin by an associated stilbene bound at the top of the hairpin (Lewis, 1997). By systematically varying the position of the guanine base within the hairpin and measuring the rate of electron transfer, a value for β, the electronic coupling parameter, could be made. Here, β was found to be intermediate between that seen in proteins, with σ bonded arrays, and that found for a highly coupled π-bonded array.

Electrochemical studies of small molecule/DNA complexes have focused primarily on solution-phase phenomena, in which DNA-induced changes in redox potentials and/or diffusion constants of organic and inorganic species have been analyzed to yield association constants (Carter, 1989, 1990; Rodriguez, 1990; Welch, 1995; Kelly, 1986; Molinier-Jumel, 1978; Berg, 1981; Plambeck, 1984). In addition, rates of guanine oxidation catalyzed by electrochemically oxidized transition-metal complexes have been used to evaluate the solvent accessibility of bases for the detection of mismatches in solution (Johnston, 1995). Electrochemical signals triggered by the association of small molecules with DNA have also been applied in the design of other novel biosensors. Toward this end, oligonucleotides have been

immobilized on electrode surfaces by a variety of linkages for use in hybridization assays. These include thiols on gold (Hashimoto, 1994a, 1994b; Okahata, 1992), carbodiimide coupling of guanine residues on glassy carbon (Millan, 1993), and alkane bisphosphonate films on Al^{3+}-treated gold (Xu, 1994, 1995). Both direct changes in mass (measured at a quartz crystal microbalance) (Okahata, 1992) and changes in current (Hashimoto, 1994a, 1994b; Millan, 1993) or electrogenerated chemiluminesence (Xu, 1994, 1995) due to duplex-binding molecules have been used as reporters for double stranded DNA. Gold surfaces modified with thiolated polynucleotides have also been used for the detection of metal ions and DNA-binding drugs (Maeda, 1992, 1994).

Other known electrochemical sensors used in an increasing number of clinical, environmental, agricultural and biotechnological applications include enzyme based biosensors. Amperometric enzyme electrodes typically require some form of electrical communication between the electrode and the active site of the redox enzyme that is reduced or oxidized by the substrate. In one type of enzyme electrode, a non-natural redox couple mediates electron transfer from the substrate-reduced enzyme to the electrode. In this scheme, the enzyme is reduced by its natural substrate at a given rate; the reduced enzyme is in turn, rapidly oxidized by a non-natural oxidizing component of a redox couple that diffuses into the enzyme, is reduced, diffuses out and eventually diffuses to an electrode where it is oxidized.

Electrons from a substrate-reduced enzyme will be transferred either to the enzyme's natural re-oxidizer or, *via* the redox-centers of the polymer to the electrode. Only the latter process contributes to the current. Thus, it is desirable to make the latter process fast relative to the first. This can be accomplished by (a) increasing the concentration of the redox centers, or (b) assuring that these centers are fast, i.e. that they are rapidly oxidized and reduced.

Most natural enzymes are not directly oxidized at electrodes without being destroyed, even if the latter are maintained at strongly oxidizing potentials. Also they are not reduced at strongly reducing potentials without being decomposed. It has, however, been shown that enzymes can be chemically modified by binding to their proteins redox couples, whereupon, if in the reduced state, they transfer electrons to

an electrode. It has also been shown that when redox polycations in solution electrostatically complex polyanionic enzymes, electrons will flow in these complexes from the substrate to the enzyme, and from the enzyme through the redox polymer, to an electrode. In addition, systems have been developed where a redox-active polymer, such as poly(vinyl-pyridine), has been introduced which electrically connects the enzyme to the electrode. In this case, the polycationic redox polymer forms electrostatic complexes with the polyanionic glucose oxidase in a manner mimicking the natural attraction of some redox proteins for enzymes, e.g., cytochrome c for cytochrome c oxidase.

The present invention provides a new approach for the detection of mismatches based on charge transduction through DNA. This electrochemical method is based on DNA-mediated electron transfer using intercalative, redox-active species and detects differences in electrical current or charge generated with fully base-paired duplexes *versus* duplexes containing a base-stacking perturbation, such as a mismatch. Carried out at an addressable multielectrode array, this method allows the processing of multiple sequences in the course of a single measurement, thus significantly improving the efficiency of screening for multiple genetic defects. Most importantly, the assay reports directly on the structural difference in base pair stacking within the hybridized duplex, rather than on a thermodynamic difference based on the condition-dependent hybridization event itself. Consequently, mismatch detection becomes independent of the sequence composition and sensors based on this approach offer fundamental advantages in both scope and sensitivity over any other existing methods.

5. SUMMARY OF THE INVENTION

The present invention provides a highly sensitive and accurate method for the detection of genetic point mutations in nucleic acid sequences and its application as a biosensor. In particular, the invention relates to electrodes that are prepared by modifying their surfaces with oligonucleotide duplexes combined with an intercalative, redox-active species and their use as sensors based on an electrochemical process in which electrons are transferred between the electrode and the redox-active species.

One aspect of the invention relates to methods for determining the presence of point mutations sequentially in a series of oligonucleotide duplexes using an intercalative, redox-active moiety. A preferred method comprises the steps of: (a) contacting at least one strand of a first nucleic acid molecule with a strand of a second nucleic acid molecule under hybridizing conditions, wherein one of the nucleic acid molecules is derivatized with a functionalized linker, (b) depositing this duplex onto an electrode or an addressable multielectrode array, (c) contacting the adsorbed duplex which potentially contains a base-pair mismatch with an intercalative, redox-active moiety under conditions suitable to allow complex formation, (d) measuring the amount of electrical current or charge generated as an indication of the presence of a base-pair mismatch within the adsorbed duplex, (e) treating the complex under denaturing conditions in order to separate the complex, yielding a monolayer of single-stranded oligonucleotides, and (f) rehybridizing the single-stranded oligonucleotides with another target sequence. Steps (c) through (f) can then be repeated for a sequential analysis of various oligonucleotide probes. Attenuated signals, as compared to the observed signals for fully base-paired, i.e. wild-type, sequences, will correspond to mutated sequences.

In some instances, it may be desirable to crosslink the intercalative, redox-active species to the duplex and perform the assay comprised of steps (a) through (d) only.

Another preferred method relates to the detection of point mutations utilizing electrocatalytic principles. More specifically, this method utilizes an electrode-bound double-stranded DNA monolayer which is immersed in a solution comprising an intercalative, redox-active species, which binds to the monolayer surface, and a non-intercalative redox-active species which remains in solution. This method comprises the steps of: (a) contacting at least one strand of a first nucleic acid molecule with a strand of a second nucleic acid molecule under hybridizing conditions, wherein one of the nucleic acid molecules is derivatized with a functionalized linker, (b) depositing this duplex which potentially contains a base-pair mismatch onto an electrode or an addressable multielectrode array, (c) immersing this complex in an aqueous solution comprising an intercalative, redox-active moiety and a non-intercalative, redox-active

moiety under conditions suitable to allow complex formation, (d) measuring the amount of electrical current or charge generated as an indication of the presence of a base-pair mismatch within the adsorbed duplex, (e) treating the complex under denaturing conditions in order to separate the complex, yielding a monolayer of single-stranded oligonucleotides, and (f) rehybridizing the single-stranded oligonucleotides with another target sequence. Steps (c) through (f) can then be repeated for a sequential analysis of various oligonucleotide probes. Utilizing this method, pronounced currents and thus increased signals will be observed due to the electrocatalytic reduction of the non-intercalative, redox-active moiety by the surface-bound, redox-active moiety.

Yet another aspect of the invention relates to a method of detecting the presence or absence of a protein and comprises the steps of: (a) contacting at least one strand of a first nucleic acid molecule with a strand of a second nucleic acid molecule under hybridizing conditions, wherein one of the nucleic acid molecules is derivatized with a functionalized linker and wherein the formed duplex is designed such to contain the recognition site of a nucleic acid-binding protein of choice, (b) depositing this duplex onto an electrode or an addressable multielectrode array, (c) contacting the adsorbed duplex with an intercalative, redox-active moiety under conditions suitable to allow complex formation, (d) potentially crosslinking the intercalative, redox-active moiety to the duplex, (e) immersing the complex in a first sample solution to be analyzed for the presence of the nucleic acid-binding protein, (f) measuring the amount of electrical current or charge generated as an indication of the presence or absence of the nucleic acid-binding protein in the sample solution, (g) treating the complex under appropriate conditions to remove the nucleic acid-binding protein, and (h) immersing it in a second sample solution to be analyzed for the presence of the nucleic acid-binding protein in order to separate the complex. Steps (e) through (h) can then be repeated for a sequential analysis of various sample solutions. Attenuated signals, as compared to signals measured for a reference solution without the nucleic acid-binding protein, indicate the presence of the nucleic acid-binding protein which is binding to its recognition site, thus causing a perturbation in base-stacking.

The invention also relates to the nature of the redox-active moieties. The requirements of a suitable intercalative, redox-active moiety include the position of its redox potential with respect to the window within which the oligonucleotide-surface linkage is stable, as well as the synthetic feasibility of covalent attachment to the oligonucleotide. In addition, chemical and physical characteristics of the redox-active intercalator may promote its intercalation in a site-specific or a non-specific manner. In a preferred embodiment, the redox-active species is in itself an intercalator or a larger entity, such as a nucleic acid-binding protein, that contains an intercalative moiety.

The nature of the non-intercalative, redox-active species for the electrocatalysis based assays depends primarily on the redox potential of the intercalative, redox-active species utilized in that assay.

Yet another aspect of the invention relates to the composition and length of the oligonucleotide probe and methods of generating them. In a preferred embodiment, the probe is comprised of two nucleic acid strands of equal length. In another preferred embodiment the two nucleic acid strands are of uneven length, generating a single-stranded overhang of desired sequence composition (i.e. a "sticky end"). The length of the oligonucleotide probes range preferably from 12 to 25 nucleotides, while the single-stranded overhangs are approximately 5 to 10 nucleotides in length. These single-stranded overhangs can be used to promote site-specific adsorption of other oligonucleotides with the complementary overhang or of enzymes with the matching recognition site.

The invention further relates to methods of creating a spatially addressable array of adsorbed duplexes. A preferred method comprises the steps of (a) generating duplexes of variable sequence composition that are derivatized with a functionalized linker, (b) depositing these duplexes on different sites on the multielectrode array, (c) treating the complex under denaturing conditions to yield a monolayer of single-stranded oligonucleotides, and (d) hybridizing these single-stranded oligonucleotides with a complementary target sequence. Another preferred method comprises the steps of (a) depositing 5 to 10 base-pair long oligonucleotide duplexes that are derivatized on one end with a functionalized linker and contain single-stranded overhangs

(approximately 5 to 10 nucleotides long) of known sequence composition at the opposite end onto a multielectrode array, and (b) contacting these electrode-bound duplexes under hybridizing conditions with single-stranded or double-stranded oligonucleotides that contain the complementary overhang.

Another aspect of the invention is directed towards the nature of the electrode, methods of depositing an oligonucleotide duplex (with or without a redox-active moiety adsorbed to it) onto an electrode, and the nature of the linkage connecting the oligonucleotide duplex to the electrode. In a preferred embodiment, the electrode is gold and the oligonucleotide is attached to the electrode by a sulfur linkage. In another preferred embodiment the electrode is carbon and the linkage is a more stable amide bond. In either case, the linker connecting the oligonucleotide to the electrode is preferably comprised of 5 to 20 σ bonds.

Yet another aspect of the invention relates to various methods of detection of the electrical current or charge generated by the electrode-bound duplexes combined with an intercalative, redox-active species. In a preferred embodiment, the electrical current or charge is detected using electronic methods, for example voltammetry or amperommetry, or optical methods, for example fluorescence or phosphoresence. In another preferred embodiment, the potential at which the electrical current is generated is detected by potentiommetry.

6. BRIEF DESCRIPTION OF DRAWINGS

Table 1 describes the electrochemical detection of single-base mismatches based on cyclic voltammograms measured for 1.0 μM daunomycin noncovalently bound to duplex-modified electrodes.

Figure 1 is a schematic diagram depicting DNA duplexes used for study of distance-dependent reduction of daunomycin. The right insert illustrates the daunomycin-guanine crosslink. The left insert shows the thiol-terminated tether which connects the duplex to the electrode surface and provides 16 σ-bonds between the electrode and the base stack.

Figure 2 illustrates cyclic voltammograms of gold electrodes modified with daunomycin-crosslinked thiol-terminated duplexes (A) SH-$^{5'}$ATGGATCTCATCTAC

+ complement and (B) SH-$^{5'}$ATCCTACTCAT**GG**AC + complement, where the bold Gs represent the daunomycin crosslinking site.

Figure 3 illustrates cyclic voltammograms of gold electrodes modified with daunomycin-crosslinked thiol-terminated duplexes containing TA and CA basepairs. The oligonucleotide SH-$^{5'}$ATTAT<u>A</u>TAATT**G**CT was hybridized with the corresponding complements containing either a T or a C opposite from the underlined A.

Figure 4 describes the charges (Q_c) measured for daunomycin at DNA-modified electrodes containing different single-base mismatches. To obtain the seven different mismatched duplexes the thiol-modified sequence, SH-$^{5'}$AGTACAGTCATCGCG, was hybridized with the following seven different complements (the mismatch is indicated in bold, and the specific basepair and the melting temperature of the duplex is given in parentheses): $^{5'}$CGCGATGACTGTACT (TA, T_m = 68 °C), $^{5'}$CGCGACGACTGTACT (CA, T_m = 56 °C), $^{5'}$CGCGATGTCTGTACT (TT, T_m = 57 °C), $^{5'}$CGCGATCACTGTACT (CC, T_m = 56 °C), $^{5'}$CGCGATGGCTGTACT (GT, T_m = 62 °C), $^{5'}$CGCGATGAATGTACT (GA, T_m = 60 °C), $^{5'}$CGCGATGCCTGTACT (CT, T_m = 58 °C).

Figure 5 describes the charge obtained for DNA-modified electrodes in the presence of 1.0 μM daunomycin. the identified duplexes of varying percentages of GC content were either fully base-paired or contained a single CA mismatch. Mismatch detection measuring the electrical current or charge generated was independent of the sequence composition.

Figure 6 describes the charges (Q_c) measured during the *in situ* detection of a CA mismatch. Electrodes were derivatized with the sequence SH-$^{5'}$AGTACAGTC<u>A</u>TCGCG, where either a C or a T was incorporated into the complement across from the underlined A. Using cyclic voltammetry, the electrochemical response of daunomycin non-covalently bound to duplex-modified electrodes was measured first for the intact TA or CA duplexes (TA *vs*. CA), secondly (after denaturation of the duplex) for the single stranded oligonucleotide (ss), thirdly (after rehybridization with the opposite complement) again for the duplex (CA *vs*.

TA), and lastly (after repeating the denaturation step) again for the single-stranded oligonucleotide (ss).

Figure 7 represents a schematic illustration of electrocatalytic reduction of ferricyanide. Methylene blue (MB^+) is reduced electrochemically through the DNA base stack to form leucomethylene blue (LB^+). Ferricyanide is then reduced by LB^+, causing the regeneration of MB^+ and the observation of catalytic currents.

Figure 8 illustrates cyclic voltammograms of gold electrodes modified with thiol-terminated duplexes containing TA and CA basepairs immersed in a solution containing 1.0 μM methylene blue and 1.0 mM ferricyanide. The oligonucleotide SH-$^{5'}$AGTACAGTCATCGCG was hybridized with the corresponding complements containing either a T or a C opposite from the underlined A.

Figure 9 shows chronocoloumetry at -350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus 20.5 μM methylene blue (pH 7) at a gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG-3' hybridized to a fully base-paired complement (upper trace) and a complement that features an A opposite the bold **C** (lower trace). The discrimination between base-paired and mismatched sequences increases with increased sampling time.

7. DETAILED DESCRIPTION OF THE INVENTION

The expression "amplification of polynucleotides" includes methods such as polymerase chain reaction (PCR), ligation amplification (or ligase chain reaction, LCR) and amplification methods based on the use of Q-beta replicase. These methods are well known and widely practiced in the art. See, e.g., U.S. Pat. Nos. 4,683,195 and 4,683,202 and Innis *et al.*, 1990 (for PCR); and Wu *et al.*, 1989a (for LCR). Reagents and hardware for conducting PCR are commercially available. Primers useful to amplify sequences from a particular gene region are preferably complementary to, and hybridize specifically to sequences in the target region or in its flanking regions. Nucleic acid sequences generated by amplification may be sequenced directly. Alternatively the amplified sequence(s) may be cloned prior to sequence analysis. A method for the direct cloning and sequence analysis of enzymatically amplified genomic segments has been described by Scharf (1986).

The term “base-stacking perturbations” refers to any event that causes a perturbation in base-stacking such as, for example, a base-pair mismatch, a protein binding to its recognition site, or any other entities that form oligonucleotide adducts.

The term “denaturing” refers to the process by which strands of oligonucleotide duplexes are no longer base-paired by hydrogen bonding and are separated into single-stranded molecules. Methods of denaturation are well known to those skilled in the art and include thermal denaturation and alkaline denaturation.

The term “hybridized” refers to two nucleic acid strands associated with each other which may or may not be fully base-paired.

The term “intercalative moieties” refers to planar aromatic or heteroaromatic moieties that are capable of partial insertion and stacking between adjacent base pairs of double-stranded oligonucleotides. These moieties may be small molecules or part of a larger entity, such as a protein. Within the context of this invention the intercalative moiety is able to generate a response or mediate a catalytic event.

The term “mismatches” refers to nucleic acid bases within hybridized duplexes which are not 100% complementary. A mismatch includes any incorrect pairing between the bases of two nucleotides located on complementary strands of DNA that are not the Watson-Crick base-pairs A:T or G:C. The lack of total homology may be due to deletions, insertions, inversions, substitutions or frameshift mutations.

The term “mutation” refers to a sequence rearrangement within DNA. The most common single base mutations involve substitution of one purine or pyrimidine for the other (e.g., A for G or C for T or vice versa), a type of mutation referred to as a "transition". Other less frequent mutations include “transversions” in which a purine is substituted for a pyrimidine, or *vice versa*, and "insertions" or "deletions", respectively, where the addition or loss of a small number (1, 2 or 3) of nucleotides arises in one strand of a DNA duplex at some stage of the replication process. Such mutations are also known as "frameshift" mutations in the case of insertion/deletion of one of two nucleotides, due to their effects on translation of the genetic code into proteins. Mutations involving larger sequence rearrangement also may occur and can be important in medical genetics, but their occurrences are relatively rare compared to the classes summarized above.

The term “nucleoside” refers to a nitrogenous heterocyclic base linked to a pentose sugar, either a ribose, deoxyribose, or derivatives or analogs thereof. The term "nucleotide" relates to a phosphoric acid ester of a nucleoside comprising a nitrogenous heterocyclic base, a pentose sugar, and one or more phosphate or other backbone forming groups; it is the monomeric unit of an oligonucleotide. Nucleotide units may include the common bases such as guanine (G), adenine (A), cytosine (C), thymine (T), or derivatives thereof. The pentose sugar may be deoxyribose, ribose, or groups that substitute therefore.

The terms “nucleotide analog”, “modified base”, “base analog”, or “modified nucleoside” refer to moieties that function similarly to their naturally occurring counterparts but have been structurally modified.

The terms “oligonucleotide” or “nucleotide sequence” refers to a plurality of joined nucleotide units formed in a specific sequence from naturally occurring heterocyclic bases and pentofuranosyl equivalent groups joined through phosphorodiester or other backbone forming groups.

The terms “oligonucleotide analogs” or “modified oligonucleotides” refer to compositions that function similarly to natural oligonucleotides but have non-naturally occurring portions. Oligonucleotide analogs or modified oligonucleotides may have altered sugar moieties, altered bases, both altered sugars and bases or altered inter-sugar linkages, which are known for use in the art.

The terms “redox-active moiety” or “redox-active species” refers to a compound that can be oxidized and reduced, i.e. which contains one or more chemical functions that accept and transfer electrons.

The term “redox protein” refers to proteins that bind electrons reversibly. The simplest redox proteins, in which no prosthetic group is present, are those that use reversible formation of a disulfide bond between to cysteine residues, as in thioredoxin. Most redox proteins however use prosthetic groups, such as flavins or NAD. Many use the ability of iron or copper ions to exist in two different redox states.

The present invention provides a highly sensitive and accurate method based on an electrochemical assay using intercalative, redox-active species to determine the

presence and location of a single or multiple base-pair mismatches. Briefly, the system is comprised of (i) a reagent mixture comprising an electrode-bound oligonucleotide duplex to which an intercalative, redox-active moiety is associated and (ii) means for detecting and quantitating the generated electrical current or charge as an indication for the presence of a fully base-paired *versus* a mismatch containing duplex. The present invention is particularly useful in the diagnosis of genetic diseases that arise from point mutations. For example, many cancers can be traced to point mutations in kinases, growth factors, receptors binding proteins and/or nuclear proteins. Other diseases that arise from genetic disorders include cystic fibrosis, Bloom's syndrome, thalassemia and sickle cell disease. In addition, several specific genes associated with cancer, such as DCC, NF-1, RB, p53, erbA and the Wilm's tumor gene, as well as various oncogenes, such as abl, erbB, src, sis, ras, fos, myb and myc have already been identified and examined for specific mutations.

The present invention provides methods for detecting single or multiple point mutations, wherein the oligonucleotide duplex carrying the redox-active species is adsorbed and therefore continuously exposed to an electrode whose potential oscillates between a potential sufficient to effect the reduction of said chemical moiety and a potential sufficient to effect the oxidation of the chemical moiety. This method is preferred over other methods for many reasons. Most importantly, this method allows the detection of one or more mismatches present within an oligonucleotide duplex based on a difference in electrical current measured for the mismatch-containing *versus* the fully base-paired duplex. Thus the method is based on the differences in base-stacking of the mismatches and is independent of the sequence composition of the hybridized duplex, as opposed to existing methods that depend on thermodynamic differences in hybridization. Furthermore, this method is nonhazardous, inexpensive, and can be used in a wide variety of applications, alone or in combination with other hybridization-dependent methods.

One particular aspect of the invention relates to the method for sequential detection of mismatches within a number of nucleic acid samples which comprises the following steps. At least one strand of a nucleic acid molecule is hybridized under suitable conditions with a first nucleic acid target sequence forming a duplex which

potentially contains a mismatch, and wherein one of the nucleic acids is derivatized with a functionalized linker. This duplex is then deposited onto an electrode or an addressable multielectrode array forming a monolayer. An intercalative, redox-active species (e.g., daunomycin) is noncovalently adsorbed (or crosslinked, if desired) onto this molecular lawn, and the electrical current or charge generated is measured as an indication of the presence of a base pair mismatch within the adsorbed oligonucleotide complex. Subsequent treatment of the duplexes containing the intercalative, redox-active species under denaturing conditions allows separation of the complex, yielding a single-stranded monolayer of oligonucleotides which can be rehybridized to a second oligonucleotide target sequence. The steps of duplex formation, adsorption of the intercalative, redox-active species, measurement of the electrical current or charge, and denaturation of the complex to regenerate the single-stranded oligonucleotides may be repeated as often as desired to detect in a sequential manner genetic point mutations in a variety of oligonucleotide probes.

The charges passed at each of the electrodes is measured and compared to the wild-type, i.e. fully base-paired, sequences. Electrodes with attenuated signals correspond to mutated sequences, while those which exhibit no change in electrical current or charge are unmutated. Furthermore, the intensity of the signal compared to the wild-type sequence not only reports the presence of the mismatch but also describes the location of the disruption within the analyzed duplex.

Another aspect of the invention relates to the method of detecting mutations utilizing electrocatalysis. Briefly, the modification of electrode surfaces with oligonucleotide duplexes provides a medium that is impenetrable by negatively charged species due to the repulsion by the high negative charge of oligonucleotides. However, electrons can be shuttled through the immobilized duplexes to redox-active intercalators localized on the solvent-exposed periphery of the monolayer, which in turn can catalytically reduce these negatively charged species. More specifically, this electrocatalytic method comprises the following steps. At least one strand of a nucleic acid molecule is hybridized under suitable conditions with a first nucleic acid target sequence forming a duplex which potentially contains a mismatch, and wherein one of the nucleic acids is derivatized with a functionalized linker. This duplex is then

deposited onto an electrode or a multielectrode array forming a monolayer. The assembly is immersed into an aqueous solution containing both an intercalative, redox-active species (e.g., methylene blue) and a non-intercalative, redox-active species (e.g., ferricyanide). The electrical currents or charges corresponding to the catalytic reduction of ferricyanide mediated by methylene blue are measured for each nucleic acid-modified electrode and compared to those obtained with wild-type, i.e. fully base-paired sequences. Subsequent treatment of the duplexes under denaturing conditions allows separation of the complex, yielding a single-stranded monolayer of oligonucleotides which can be rehybridized to a second oligonucleotide target sequence. The steps of duplex formation, measurement of the catalytically enhanced electrical current or charge, and denaturation of the complex to regenerate the single-stranded oligonucleotides may be repeated as often as desired to detect in a sequential manner genetic point mutations in a variety of oligonucleotide probes. This particular method based on electrocatalysis at oligonucleotide-modified surfaces is extremely useful for systems where attenuated signals resulting from the presence of mismatches are small. The addition of a non-intercalative electron acceptor amplifies the signal intensity, and allows more accurate measurements. This approach may be particularly useful to monitor assays based on redox-active proteins which bind to the oligonucleotide-modified surface, but are not easily oxidized or reduced because the redox-active center is not intercalating.

The present invention further relates to the nature of the redox-active species. These species have a reduced state in which they can accept electron(s) and an oxidized state in which they can donate electron(s). The intercalative, redox-active species that are adsorbed or covalently linked to the oligonucleotide duplex include, but are not limited to, intercalators and nucleic acid-binding proteins which contain a redox-active moiety.

An intercalator useful for the specified electrochemical assays is an agent or moiety capable of partial insertion between stacked base pairs in the nucleic acid double helix. Examples of well-known intercalators include, but are not limited to, phenanthridines (e.g., ethidium), phenothiazines (e.g., methylene blue), phenazines (e.g., phenazine methosulfate), acridines (e.g., quinacrine), anthraquinones (e.g.,

daunomycin), and metal complexes containing intercalating ligands (e.g., phi, chrysene, dppz). One example of a suitable metal complex is the octahedral metal complex, $Ir(bpy)(phen)(phi)^{3+}$. Some of these intercalators may interact site-selectively with the oligonucleotide duplex. For example, the chrysene ligand is known to intercalate at the mispaired site of a duplex itself (Jackson, 1997), which can be exploited for selective localization of an intercalator. This can be in particular useful to construct a duplex monolayer which contains the intercalative, redox-active species exclusively at its periphery.

In the case of redox-active nucleic acid-binding proteins, differences in DNA-mediated electron transfer between the duplex-bound protein and the electrode allow for the detection of base-pair mismatches or other base-stacking perturbations. Examples of redox-active proteins include, but are not limited to, mut Y, endonuclease III, as well as any redox-active cofactor-containing DNA-binding protein. Such proteins convert to one of an oxidized and reduced form upon reacting with a selected substrate, whereafter the operation of the electrode regenerates the other of the oxidized and reduced forms.

The choice of a protein depends partially on its adsorption and binding properties to biological macromolecules, i.e. nucleic acids, and with non-biological macromolecules, whether in a homogeneous solution, or when immobilized on a surface. By changing the absorption or binding characteristics, selectivity, signal to noise ratio and signal stability in these assays can be improved. The charge of a protein affects its adsorption on surfaces, absorption in films, electrophoretic deposition on electrode surfaces, and interaction with macromolecules. It is, therefore, of importance in diagnostic and analytical systems utilizing proteins to tailor the charge of the protein so as to enhance its adsorption or its binding to the macromolecule of choice, i.e the nucleic acid. In other cases, i.e when the detection assay is used during several cycles, it is of equal importance to be able to facilitate desorption, removal, or stripping of the protein from the macromolecule. These assays require oligonucleotide duplexes that are designed such as to allow for site-specific binding of the protein of choice, which may require a single-stranded

overhang. Once the protein is adsorbed onto the nucleic acid, electrons are relayed *via* the oligonucleotide duplex to the electrode.

The nature of the non-intercalative, redox-active species used in a particular electrocatalytic assay depends primarily on the redox potential of the intercalating, redox-active species utilized in that same assay. Examples include, but are not limited to, any neutral or negatively charged probes, for example ferricyanide/ferrocyanide, ferrocene and derivatives thereof (e.g., dimethylaminomethyl-, monocarboxylic acid-, dicarboxylic acid-), hexacyanoruthenate, and hexacyanoosmate.

Yet another aspect of the invention relates to a method of detecting the presence or absence of a protein inducing base-stacking perturbations in DNA duplexes, this method comprising the following steps. At least one strand of a nucleic acid molecule is hybridized under suitable conditions with a second strand of nucleic acid molecule forming a duplex, wherein one of the nucleic acids is derivatized with a functionalized linker. This duplex is designed such to contain the recognition site of a protein of choice at a distinct site along that duplex. This duplex is then deposited onto an electrode or an addressable multielectrode array forming a monolayer and an intercalative, redox-active species is adsorbed onto this molecular lawn. In a preferred embodiment, the intercalative, redox-active species is site-specifically localized. In another preferred embodiment, the intercalative, redox-active species is crosslinked to the oligonucleotide duplex. This formed complex is then exposed to a sample solution that potentially contains the specific protein and the electrical current or charge generated is measured as an indication of the presence or absence of the protein. Subsequently, the protein is removed under appropriate conditions to regenerate the duplex containing the intercalative, redox-active moiety. The steps of duplex formation, adsorption or crosslinking of the intercalative, redox-active species, measurement of the electrical current or charge, and regeneration of the duplex containing the intercalative, redox-active moiety may be repeated as often as desired to detect in a sequential manner the presence of a specific protein in multiple sample solutions.

The charges passed at each of the electrodes are measured and compared to the charges measured in a reference solution without the protein. Electrodes with

attenuated signals indicate the presence of the protein in question which is binding to its recognition site, thus causing a perturbation in base-stacking. Examples of proteins that can be used for this assay include, but are not limited to, restriction enzymes, TATA-binding proteins, and base-flipping enzymes (e.g., DNA methylase).

The present invention also relates to the choice of nucleic acid probes. Any nucleic acid, DNA or RNA, can be subjected to this mismatch detection method, provided that the mismatch(es) to be detected lie within the region between the attachment site of the intercalative, redox-active moiety and the electrode in order to be able to measure a difference in electrical current. The nucleic acid probes to be compared may comprise natural or synthetic sequences encoding up to the entire genome of an organism. These probes can be obtained from any source, for example, from plasmids, cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, organelles and higher organisms such as plants and animals. The samples may be extracted from tissue material or cells, including blood cells, amniocytes, bone marrow cells, cells obtained from a biopsy specimen and the like, by a variety of techniques as described for example by Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Cold Spring Harbor, New York (1982), incorporated herein by reference.

Alternatively, the sequences of choice can also be prepared by well known synthetic procedures. For standard DNA and RNA synthesis methods, see for example "Synthesis and Applications of DNA and RNA" ed. S. A. Narang, Academic Press, 1987, M. J. Gait, "Oligonucleotide Synthesis", IRL Press, Wash. D.C. U.S.A., 1984, and “Oligonucleotides and Analogues” ed. F. Eckstein, IRL Press, Wash. D.C. U.S.A., 1991, as incorporated herein by reference. Briefly, oligonucleotides and oligonucleotide analogs may be synthesized, conveniently through solid state synthesis of known methodology. In a preferred embodiment, the monomeric units are added to a growing oligonucleotide chain which is covalently immobilized to a solid support. Typically, the first nucleotide is attached to the support through a cleavable linkage prior to the initiation of synthesis. Step-wise extension of the oligonucleotide chain is normally carried out in the 3' to 5' direction. When the synthesis is complete, the polymer is cleaved from the support by hydrolyzing the

linkage mentioned above and the nucleotide originally attached to the support becomes the 3' terminus of the resulting oligomer. Nucleic acid synthesizers such as the Applied Biosystems, Incorporated 380B are commercially available and their use is generally understood by persons of ordinary skill in the art as being effective in generating nearly any oligonucleotide or oligonucleotide analog of reasonable length which may be desired. Triester, phosphoramidite, or hydrogen phosphonate coupling chemistries are used with these synthesizers to provide the desired oligonucleotides or oligonucleotide analogs.

In addition, the invention also relates to nucleic acid probes that are constructed with a defined sequence comprised of nucleotide and non-natural nucleotide monomers to restrict the number of binding sites of the intercalative, redox-active agent to one single site. For example, in the case of the redox-active intercalator daunomycin mixed nucleotide/non-natural nucleotide oligomers were prepared containing A-T and/or I-C basepairs and one discrete guanine binding site to which daunomycin is crosslinked. The non-natural nucleotides are constructed in a step-wise fashion to produce a mixed nucleotide/non-natural nucleotide polymer employing one of the current DNA synthesis methods well known in the art, see for example "Synthesis and Applications of DNA and RNA" ed. S. A. Narang, Academic Press, 1987, M. J. Gait, "Oligonucleotide Synthesis", IRL Press, Wash. D.C. U.S.A., 1984, and "Oligonucleotides and Analogues" ed. F. Eckstein, IRL Press, Wash. D.C. U.S.A., 1991.

Methods and conditions used for contacting the oligonucleotide strands of two DNAs, two RNAs or one DNA and one RNA molecule under hybridizing conditions are widely known in the art. Suitable hybridization conditions may be routinely determined by optimization procedures well known to those skilled in the art to establish protocols for use in a laboratory. See e.g., Ausubel *et al.*, *Current Protocols in Molecular Biology, Vol. 1-2*, John Wiley & Sons (1989); Sambrook *et al.*, Molecular Cloning A Laboratory Manual, 2nd Ed., Vols. 1-3, Cold Springs Harbor Press (1989); and Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Cold Spring Harbor, New York (1982), all of which are incorporated by reference herein. For example, conditions such as temperature,

concentration of components, hybridization and washing times, buffer components, and their pH and ionic strength may be varied.

Another aspect of the invention relates to a surface-modified electrode and its use in a bioelectrochemical process, in which electrons are transferred directly between an electrode and an electroactive biological material which is capable of accepting or donating one or more electrons. Such a bioelectrochemical process can be in either direction. In particular, the invention provides electrodes having their surface modified with oligonucleotide duplexes carrying an intercalative, redox-active moiety. The electrode can be of any material compatible with the surface modifier being adsorbed or bound thereon, including, but not limited to noble metals such as gold, silver, platinum, palladium, as well as carbon. The preferred material for the electrodes are gold and carbon.

The oligonucleotide duplex can be adsorbed onto the electrode in any convenient way. Preferably, the process of preparing such a modified electrode comprises adsorbing the oligonucleotide duplex which is derivatized at the 5'-end with a functionalized linker chains onto the electrode surface in one monolayer to obtain a uniform lawn. These linkers include, but are not limited to, thiol- or amine-terminated chains. This process is generally understood by persons of ordinary skill in the art as and is relatively simple, reproducible and can easily be automated.

Furthermore, the density and composition of the monolayer is subject to variation depending on the selected assay. Methods of detecting single base-pair mismatches using intercalative, redox-active moieties require a densely packed monolayer to prevent the adsorbed intercalative, redox-active moieties from diffusing into the lawn. The method for detecting the presence or absence of a protein requires preferably an uneven monolayer comprised of duplexes of variable length to allow the protein to bind effectively to its recognition site along the duplex.

In addition, the present invention further relates to methods of creating a spatially addressable array of adsorbed duplexes. In a preferred embodiment, oligonucleotide duplexes of variable sequence composition that are derivatized at the 5'-end with a functionalized linker are deposited onto a multielectrode array. Subsequent treatment of these electrode-bound duplexes under denaturing conditions

yields a monolayer of single-stranded oligonucleotides, which can then be hybridized with a complementary oligonucleotide probe that potentially contains a mismatch. In another preferred embodiment, short oligonucleotide duplexes (5 to 10 base-pairs in length) that are derivatized on one end with a functionalized linker and contain single-stranded overhangs (5 to 10 nucleotides in length) of designed sequence composition at the opposite end are deposited onto a multielectrode array to generate a spatially addressable matrix. These electrode-bound duplexes can then be hybridized with single-stranded or double-stranded oligonucleotides that contain the complementary overhang.

Solid supports containing immobilized molecules have been extensively used in research, in clinical analyses and in the commercial production of foods and chemicals (see e.g., U.S. Pat. No. 5.153,166; Akashi, 1992). Immobilized nucleic acids are used in hybridization assays (Lamture, 1994) and immobilized proteins in radioimmuno or ELISA assays (see, U.S. Pat. No. 5,314,830). In addition, enzymes have been immobilized to facilitate their separation from product and to allow for their efficient and repetitive use. A number of important factors have to be considered in the development of an effective immobilization procedure. First, the procedure must minimize non-specific adsorption of molecules. Second, the procedure must maintain the functional integrity of the immobilized molecules. Third, the stability of the bond between the support and the immobilized molecule must be such to avoid leaching which would lead to reduced accuracy and sensitivity. Finally, the coupling procedure must be efficient enough to result in a support with a high capacity for the target molecules as well as be cost effective.

Another aspect of the invention relates to measuring the electrical current as a function of degree of hybridization of the oligonucleotide duplex adsorbed onto the electrode. When the intercalative, redox-active species is exposed to electrochemical or chemical energy, the electrical current may be continuously detected using techniques well known in the art. These include, but are not limited electronic methods, for example voltammetry or amperommetry, or optical methods, for example fluorescence or phosphoresence.

Generally, photoluminescence excitation and emission occur with electromagnetic radiation of between about 200 nanometers and about 900 nanometers in wavelength. Likewise, chemiluminescent and electrochemiluminescent emission generally occur with the emitted electromagnetic radiation being between about 200 nanometers and about 900 nanometers in wavelength. The potential at which the reduction or oxidation of the chemical moiety occurs depends upon its exact chemical structure as well as factors such as the pH of the solution and the nature of the electrode used. It is well known how to determine the optimal emission and excitation wavelengths in a photoluminescent system and the optimal potential and emission wavelength of an electrochemiluminescent and chemiluminescent system.

There are many methods for quantifying the amount of luminescent species present. The rate of energy input into the system can provide a measure of the luminescent species. Suitable measurements include, for example, measurements of electric current when the luminescent species is generated electrochemically, the rate of reductant or oxidant utilization when the luminescent species is generated chemically or the absorption of electromagnetic energy in photoluminescent techniques. In addition, the luminescent species can be detected by measuring the emitted electromagnetic radiation. All of these measurements can be made either as continuous, event-based measurements, or as cumulative methods which add the signal over a long period of time. Event-based measurements may be carried out with photomultiplier tubes, photodiodes or phototransistors to produce electric currents proportional in magnitude to the incident light intensity, or by using charge couple devices. Examples of cumulative methods are the integration of event-based data, and the use of photographic film to provide cumulative data directly.

The publications and other reference materials referred to herein describe the background of the invention and provide additional detail regarding its practice and are hereby incorporated by reference. For convenience, the reference materials are referenced and grouped in the appended bibliography.

The present invention is further described in the following examples. These examples are for illustrative purposes only, and are not to be construed as limiting the scope of the invention as set forth in the appended claims.

Examples

Materials. Phosphoramidite reagents (including the C_6S-S thiol modifier) were obtained from Glen Research. [γ-^{32}P]dATP was obtained from NEN-DuPont. Potassium ferrocyanide (Fisher) was recrystallized from aqueous solution prior to use. Daunomycin was obtained from Fluka.

Synthesis of Derivatized Duplexes. Oligonucleotides immobilized on a controlled pore glass resin were treated in succession with carbonyldiimidazole and 1,6-diaminohexane (1g/10ml dioxane, 30 min/ea.) at the 5'-hydroxy terminus before cleavage from the resin (Wachter, 1986). After deprotection, the free amine was treated with 2-pyridylthiopropionic acid N-succinimide ester to produce a disulfide (Harrison, 1997). The sequences were purified by reverse-phase HPLC, converted to free thiols using dithiothreitol, and repurified before hybridization to their complements. Derivatized oligonucleotides were characterized by mass-assisted laser desorption ionization time-of-flight mass spectrometry and HPLC retention times. Duplexes were hybridized in deoxygenated 5 mM phosphate/50 mM NaCl (pH 7) by heating to 90 °C followed by slow cooling to room temperature. Unprotected duplexes were stored frozen under argon to prevention oxidation of the thiol.

Atomic Force Microscopy (AFM). All AFM images were collected using a MultiMode AFM running on the NanoScope IIIa controller (Digital Instruments, Santa Barbara, CA). A glass AFM chamber (Digital Instruments, Santa Barbara, CA) and a fluid volume of approximately 50 microliters were used for the experiments. Si_3N_4 cantilevers (spring constant, 0.06 N/m) with integrated, end-mounted oxide-sharpened Si_3N_4 probe tips were used. The applied vertical force of the AFM probe during imaging was minimized to beneath 100pN. Continually adjusting the cantilever deflection feedback setpoint compensated for thermal drifting of the cantilever and a consistent, minimum force was maintained AFM height calibrations were carried out on a NIST-traceable 180-nm height standard and then confirmed by measuring a single-atom step in the Au gold surface. The AFM images were recorded

in "Height" (or constant force) mode. Holes in the monolayer used to determine monolayer thicknesses were prepared by decreasing the scan size to approximately 100-150 nm, increasing the scan rate to 24-30 Hz, and increasing the vertical force by advancing the setpoint several units. After about one minute, the scan size, scan rate, and setpoint were returned to their previous values, and images featuring a bare gold square were captured. All images captured for height-contrast analysis were recorded at minimum vertical tip forces. This was accomplished by decreasing the set-point until the tip disengaged from the surface, then reintroducing it with the minimum force required to achieve a stable image. In several cases, the film height was also measured in tapping mode, and gave the same result as the contact-mode experiments.

Electrochemistry. Cyclic voltammetry (CV) was carried out on 0.02 cm^2 polycrystalline gold electrodes using a Bioanalytical Systems (BAS) Model CV-50W electrochemical analyzer at 20 ± 2 °C in 100 mM phosphate buffer (pH 7). A normal three-electrode configuration consisting of a modified gold-disk working electrode, a saturated calomel reference electrode (SCE, Fisher Scientific), and a platinum wire auxiliary electrode was used. The working compartment of the electrochemical cell was separated from the reference compartment by a modified Luggin capillary. Potentials are reported versus SCE. Heterogeneous electron-transfer rates were determined and analyzed by CV (Nahir, 1994; Weber, 1994; Tender, 1994).

Ellipsometry. Optical ellipsometry ($\lambda = 632.8$ nm) was carried out on dried samples at 25 °C using a Gaertner Model L116C ellipsometer.

Example 1: Site-specific Incorporation of a Redox-Active Intercalator into a DNA Duplex.

The redox-active intercalator daunomycin (DM) (Arcamone, 1981) was incorporated into the DNA duplex to investigate charge transduction through these duplexes (Figure 1). DM undergoes a reversible reduction (Molinier-Jumel, 1978; Berg, 1981) within the potential window of the monolayers (Kelley, 1997a), and covalent adducts of intercalated DM crosslinked to the 2-amino group of guanine (Leng, 1996) have been crystallographically characterized within duplex DNA (Wang, 1991). Thus, a series of oligonucleotides primarily containing A-T or inosine (I)-C pairs were constructed with discrete guanine binding sites to which DM was

crosslinked. Preferably, thiol-terminated duplexes (0.1 mM) containing an adjacent pair of guanines were hybridized, incubated with 0.2 % formaldehyde and 0.2 mM DM in 5 mM phosphate, 50 mM NaCl, pH 7 for 1 h, and phenol extracted to remove excess DM.

Moving the guanine site along the duplex resulted in a systematic variation of the through-helix DM/gold separation, and allowed an investigation of the effect of distance on the dynamics of charge transport through the monolayers (Figure 1).

Example 2: Characterization of DNA Duplexes Modified with a Redox-Active Intercalator.

Modified duplexes were characterized by mass spectrometry, ultraviolet/visible absorption spectroscopy, and thermal denaturation experiments, all of which were consistent with a 1:1 DM-duplex stoichiometry. For example, the duplex SH-$(CH_2)CONH(CH_2)_6NHCO_2$-$^{5'}$ATCCTACTCATGGAC with its inosine complement modified with DM was analyzed by MALDI-TOF spectrometry. Mass-to-charge ratios (found/calc) of 5284/(5282) (DM + SH strand), 4541/(4540) (complement), and 4742/(4742) (SH strand) were detected. These values correspond to the calculated masses for fragments expected from this duplex. UV-visible absorption spectroscopy also revealed a 1:1 duplex/DM stoichiometry based upon comparison of the duplex absorbance at 260 nm ($\varepsilon = 14.9 \times 10^3\ M^{-1}\ cm^{-1}$) and the absorbance of intercalated DM at 480 nm ($\varepsilon = 5.1 \times 10^3\ M^{-1}\ cm^{-1}$). In the presence of 100 mM phosphate, 100 mM $MgCl_2$, and at pH 7, thermal denaturation studies of 5 DM duplexes (monitored by absorbance at 260 nm) revealed melting temperatures of 48 and 50°C for the native and daunomycin-crosslinked duplexes, respectively. A similar melting profile was obtained by monitoring hypochromicity at 482 nm for the DM duplex.

Example 3: Preparation of Gold Electrodes Derivatized with DNA Duplexes.

Electrodes were conveniently prepared by modifying gold surfaces with 15 base-pair DNA duplexes derivatized at the 5' end with a thiol-terminated alkane chain. Bulk gold electrodes were polished successively with 0.3- and 0.5-μM alumina (Buhler), sonicated for 30 min, and etched in 1.0 M sulfuric acid. Au(111) surfaces

were prepared by vapor deposition onto mica or glass (Widrig, 1991; Zei, 1983). Electrodes were then modified by incubation in 0.1 mM solutions of derivatized DNA duplexes in 5 mM phosphate/50 mM NaCl (pH 7) for 12 - 48 h at ambient temperature. Modified electrodes were rinsed in buffer prior to use.

Before deposition of the duplexes onto the gold surfaces, the presence of the free thiols was confirmed using a spectroscopic assay based on dithionitrobenzene (Riddles, 1979). Subsequently, the samples were deposited onto the gold surfaces for 12 - 24 h.

Electrochemical assays, radioactive tagging experiments, and atomic force microscopy (AFM) (Kelley, 1997a, 1997b) all indicate that the oligonucleotides form densely packed monolayers oriented in an upright position with respect to the gold surface.

Example 4: Characterization of Modified DNA Duplexes Monolayers on Gold Surfaces

The DM-modified duplexes readily formed self-assembled monolayers on gold. AFM studies of modified films reveal densely packed monolayers with heights greater than 45 Å at open circuit. More specific, AFM studies were carried out under electrochemical control, and revealed that the DNA films undergo a potential-dependent change in structure. At open circuit, the monolayer film height is 45(3) Å. Based on the anisotropic dimensions of the 15-base pair duplexes (20 Å in diameter vs. 60 Å in length), this thickness indicates that the helical axis is oriented ~ 45" from the gold surface. At applied voltages negative of the potential of zero charge, film thickness of ~60 Å are observed; more positive potential cause a drop in the film height to a limiting value of 20 Å at low surface coverages.

Based on the crossectional area of DNA (~ 3 nm^2) and the geometrical area of the gold electrodes (0.02 cm^2), the maximum surface coverage of DNA was calculated as ~6×10^{-11} mol/cm^2. Coulometry at electrodes modified with duplexes containing crosslinked DM revealed a DM surface coverage of $7.5(7) \times 10^{-11}$ mol/cm^2, indicating that the surface is densely packed with the modified duplexes. The DM value appeared to exceed slightly the theoretical Γ for DNA, and likely resulted from additional electrode surface roughness.

To assess routinely the surface coverage of DM-derivatized DNA on gold, the electrochemical response of $Fe(CN)_6^{4-}$ (2 mM) was monitored. This negatively charged ion is repelled from the modified-electrode surface by the polyanionic DNA, and exhibits essentially no response when the surface is well covered. While not a direct measure of surface coverage, this technique allowed the convenient assay of individual electrodes for adequate modification.

Cyclic voltammograms of these surfaces showed the reversible reduction of DM at -0.65 V versus SCE (Molinier-Jumel, 1978; Berg, 1981). These films were extremely stable and exhibited responses characteristic of surface-bound species (*e.g.*, linear plots of peak current versus scan rate) (Bard, 1980).

Example 5: Measurement of Electrochemical Response of a Redox-Active Intercalator Crosslinked to a *Fully Base-Paired* DNA Duplex on a Gold Surface.

Integration of the electrochemical response yielded a surface coverage (Γ) of electroactive daunomycin of 7.5(7) x 10^{-11} mol/cm^2, a value in good agreement with the coverages of 15-base pair duplexes previously measured via ^{32}P labeling (Kelley, 1997a). However, significant fluctuations in the surface coverages of DM-modified duplexes were observed. Therefore, only electrodes which exhibited both large integrated currents for the reduction of crosslinked DM *and* an attenuated responses for the oxidation of ferrocyanide in solution were studied.

Given the 1:1 stoichiometry of crosslinked DM to DNA, the observed data indicated that all of the bound DM was electrochemically reduced. Doping these films with increasing percentages of DM-free duplexes resulted in a linear decrease in the observed electrochemical signals (as determined from coulometric assays), consistent with each of the bound intercalators being electrochemically active.

Remarkably, efficient reduction of DM was observed regardless of its position along the 15-base-pair sequence as illustrated in Figure 2. Based on molecular modeling, the DM/gold separations span ~ 25 Å. The through-helix DM-electrode separation is > 10 Å for DM bound at the end of the duplex closest to the electrode (Figure 2A), and the DM-electrode separation is > 35 Å (Figure 2B) for DM crosslinked to the end of the duplex farthest from the electrode. The surface coverage

of electroactive daunomycin for these 15 base-pair duplexes as measured by integrating the currents within the illustrated voltammograms were 0.65×10^{-10} mol/cm^2 and 0.80×10^{-10} mol/cm^2, respectively. The DM:DNA stoichiometry for these same samples, measured by absorption spectroscopy were 0.9:1 and 1.1:1, respectively. Thus, the charge did not depend on distance, but did reflect the yield of crosslinking.

Example 6: Measurement of Electrochemical Response of a Redox-Active Intercalator Crosslinked to a *Mismatch-Containing* DNA Duplex on a Gold Surface.

Electrochemical responses of a redox-active intercalator crosslinked to a *mismatch-containing* DNA duplex on a gold surface were measured to determine whether these observed rates were a result of direct contact between the redox-active cofactor and the electrode surface (which has previously been shown to yield apparently distance-independent heterogeneous electron transfer (Feng, 1995, 1997)). A single site within the 15-base-pair duplex was mutated to produce a CA mismatch (known to cause local disruptions in the DNA base stack (Patel, 1984; Aboul-ela, 1985) between the intercalated DM and the electrode surface. Figure 3 illustrates that such a simple change virtually eliminated the electrochemical response.

The coulometry of DM at electrodes modified with CA-containing duplexes varied to some degree as a function of the surface coverage. At high surface coverages (as determined by the ferrocyanide assay), essentially no signal was observed with the mismatched duplexes. However, at more moderate surface coverages, small signals corresponding to the reduction of DM were found. These typically did not exceed 30 % of the signals found for the TA duplexes. The morphology of partial DNA monolayers is unknown.

Significantly, sequences in which the positions of the DM and CA mismatch were reversed (such that the mismatch was located above the DM relative to the gold) showed no diminution in the electrochemical response. AFM images of the CA-mutated sequences were identical to those of the TA analogs (monolayer thicknesses of ~ 40Å at open circuit), revealing that the bulk structure of the DNA films was not significantly altered by the presence of a mismatch. Moreover, the oxidation of

ferrocyanide was similarly attenuated at both surfaces. Expected masses for DM-crosslinked DNA duplexes (accounting for the single base change) were measured by mass spectrometry, and spectrophotometric assays revealed that the extent of crosslinking was identical in both fully paired and mismatched sequences.

The exquisite sensitivity of the electrochemistry of DM to intervening lesions in the base stack provides therefore the basis for an exceptionally versatile DNA-mismatch sensor.

Example 7: Analysis of the Electrochemical Behavior of *Fully Base-Paired* or *Mismatch-Containing* DNA Duplexes Containing Non-Crosslinked Intercalators.

A practical method to detect mismatches utilizes a system based on non-crosslinked, intercalative, redox-active species. The electrochemistry of DM non-covalently intercalated into DNA-modified films was studied in order to develop a general approach to test heterogeneous sequences that may possess more than one guanine-binding site. Coulometric titrations confirmed that DM strongly binds to surfaces modified with fully base-paired duplexes, and yielded affinity constants very similar to those determined for homogeneous solutions (Arcamone, 1981; Molinier-Jumal, 1978; Berg, 1981). At bulk DM concentrations ≥1 μM, the modified electrodes were saturated with intercalator, and hold approximately one intercalator per surface-confined duplex. Furthermore, intercalators non-covalently bound to these films exhibited electrochemical properties quite similar to those described for crosslinked DM, with the exception that the binding was reversible, i.e. in pure buffer solutions, decreasing voltammetric signals were observed until total dissociation was evident.

In accord with the studies of covalently bound DM, incorporation of a single CA mismatch into these duplexes dramatically decreased the electrochemical response (Table 1). The magnitude of this mismatch effect depended strongly on the location of the CA base step along the sequence: when the mutation was buried deep within the monolayer, the measured charge drops by a factor-of 3.5(5) (relative to the Watson-Crick duplex), but by only 2.3(4) when it was located near the solvent-exposed terminus. These observations were consistent with DM occupying sites near

the top of the densely packed monolayer, as suggested in earlier studies of methylene blue bound to these same surfaces (Kelley, 1997b). The intensity of the electrochemical signals therefore not only reports the presence of the mismatch but also may describe the location of the disruption.

In addition, lateral charge diffusion within these monolayers was analyzed. For example, a series of fully base-paired films (sequence: SH-$^{5'}$AGTACAGTCATCGCG) doped with increasing fractions of CA-mismatched helices were prepared (the mismatch was localized at the base step denoted by the bold C in the above sequence.) The coulometric response of DM non-covalently bound to these surfaces was strongly dependent on the film composition such that the electrochemical signals decreased linearly with increasing percentages of mutated duplexes. As there is no measurable difference in the affinities of DM toward TA- versus CA-containing films, this linear response indicated that the electroinactive intercalators (presumably those molecules bound to mutated helices) are not reduced by lateral charge transfer from the electroactive species. This result further supports a through-helix pathway for charge transduction, as intermolecular interactions between intercalators bound to different duplexes in the film evidently do not mediate efficient electron transfer.

Example 8: Analysis of Mutation Dependence of Electrochemical Response.

To explore the scope of this mismatch detection strategy, the charge (Q_c) for DM at DNA-modified electrodes containing different single-base mismatches was analyzed (Figure 4). The seven different mismatched duplexes were obtained by hybridization of the thiol-modified sequence, SH-$^{5'}$AGTACAGTCATCGCG, with the following seven different complements (the mismatch is indicated in bold, and the specific basepair and the melting temperature of the duplex is given in parentheses): $^{5'}$CGCGATGACTGTACT (TA, T_m = 68 °C), $^{5'}$CGCGACGACTGTACT (CA, T_m = 56 °C), $^{5'}$CGCGATGTCTGTACT (TT, T_m = 57 °C), $^{5'}$CGCGATCACTGTACT (CC, T_m = 56 °C), $^{5'}$CGCGATGGCTGTACT (GT, T_m = 62 °C), $^{5'}$CGCGATGAATGTACT (GA, T_m = 60 °C), $^{5'}$CGCGATGCCTGTACT (CT, T_m = 58 °C). The charges were then calculated by integrating background-subtracted cyclic voltammograms. The

obtained values were based on > 5 trials, and the results were comparable for experiments run side-by-side or utilizing different sample preparations. The melting temperatures of the oligomers in solution were measured by monitoring duplex hypochromicity at 260 nm using samples that contained 10 μM duplex, 100 mM $MgCl_2$, and 100 mM phosphate at pH 7.

Coulometric analysis confirmed that the attenuation of the characteristic DM response was strongly dependent upon the identity of the mutation. In general, pyrimidine-pyrimidine and purine-pyrimidine mismatches caused marked decreases in the electrochemical signals; the one purine-purine pair studied (a GA mismatch, which is notoriously well-stacked within duplex DNA (Patel, 1984; Aboul-ela, 1985)) did not show a measurable effect. Suprisingly, a significant decrease was caused by a GT pair, which is also not highly disruptive to the helix. This wobble base pair, although thermodynamically stable, appears to mediate electron transfer poorly.

Figure 4 illustrates that across a very narrow range of duplex thermal stabilities, large differences in the electrochemical response were observed. Overall, the electrochemical properties of films containing the different mismatches correlated with the degree of disruption to base stacking with the individual duplexes. These results underscore the sensitivity of this electrochemical assay to base stacking within DNA, and demonstrate the viability of detecting mismatches based upon charge transduction through thin films.

Example 9: Analysis of Sequence Dependence of Mismatch Detection Assay.

A single CA mismatch was incorporated into three different DNA duplexes to test for the sequence dependence of the assay. The duplexes featured varying percentages of GC content, representing a wide range of duplex stabilities. The melting temperatures for these duplexes, as determined by thermal denaturation measurements obtained by monitoring hypochromicity at 260 nm in duplex solutions containing 10 μM duplex, 100 mM phosphate, and 100 mM $MgCl_2$ were: (SH-$^{5'}$-ATATAATATATGGAT): TA = 47 °C, CA = 32 °C; (SH-$^{5'}$-AGTACAGTCATCGCG): TA = 68 °C, CA = 56 °C; (SH-$^{5'}$-GGCGCCCGGCGCCGG): GC = 82 °C, CA = 69 °C. The charge was quantitated

from integrating background-subtracted cyclic voltammograms obtained at $\upsilon = 100$ mV/s and was corrected for electrode area. As illustrated in Figure 5, the characteristic drop in coulometric signals for DNA duplexes containing a single CA mismatch compared to fully base-paired DNA films was essentially invariant across AT-rich to GC-rich sequences. This sequence-independent response is not achievable using traditional mismatch detection assays based upon differential hybridization.

Example 10: Analysis of Electrochemical Response During Repeated Cycles.

To extend this methodology to single-stranded targets, techniques for *in situ* hybridization were developed. Thiol-modified duplexes were deposited on the gold surface, heat denatured, thoroughly rinsed, then rehybridized with the desired target by incubation in ≥ 50 pmol of single-stranded oligonucleotide. The electrochemical properties of the resulting surfaces were identical to those described above, suggesting the suitability of this system for genomic testing.

For example, a 15-base-pair oligonucleotide, $^{5'}$AGTACAGTC<u>A</u>TCGCG, which was derivatized with a thiol-terminated linker, was hybridized both to its native complement and to a mutated complement (at the site underlined in the sequence), generating a fully base-paired duplex and a CA mismatch-containing duplex, respectively (Figure 6). These duplexes were deposited on separate electrodes and the electrochemical responses of DM non-covalently bound to these duplexes were measured using cyclic voltammetry ($\nu = 100$ mV/s, 1.0 μM DM). Figure 6 illustrates that DM exhibited electrochemical responses characteristic of fully base-paired and CA-mutated films, respectively. The surfaces were then denatured by immersing the electrodes in 90 °C pure buffer for 2 min to yield single-stranded monolayers of identical sequence. Cyclic voltammetry of DM at these electrodes now revealed nearly identical responses, with the reduction appearing highly irreversible, broadened, and becoming smaller as a function of increasing scans. Importantly, the electrode that initially possessed the CA mismatch displayed a large signal (for the first scan) after denaturation, while the reverse was true for the corresponding TA analog. New duplexes were formed by incubating the electrodes with 100 pmol of the opposite complement in the presence of buffered 100 mM $MgCl_2$ such that the

complements were traded (TA → CA, CA → TA), and the electrochemistry at the duplex-modified films again showed the characteristic behavior expected for fully base-paired and CA-mutated films. Finally, the electrodes were again heated to denature the duplexes and quantitation of the response showed again the characteristics for single-stranded oligonucleotides. Thus, electrodes can be cycled through this sequence of events repeatedly, indicating a practical means to detect point mutations within natural DNAs.

Example 11: Detection of Genetic Mutations Within a Specific Region of the p53 Gene Using Direct Current Measurement of Thiol-Modified Duplexes on Gold Surfaces .

A specific embodiment utilizes a gold-microelectrode array with approximately thirty addressable sites. A different 20-base pair duplex derivatized with a hexylthiol linker is attached to each of these sites by deposition form a concentrated duplex solution overnight. The sequences are chosen to correspond to the 600-base pair region within exons 5 through 8 of the p53 gene where most of the cancer-related mutations are found. The array is immersed in aqueous solution at 90 °C for 60 seconds to denature the immobilized duplexes and remove the complementary strands. The human sample containing the p53 gene is fragmented either before or after amplification. A solution containing the fragmented genomic single-stranded DNA is deposited on the array for one hour to allow hybridization to occur. Then, in the presence of a 1.0 μM DM solution, the charge passed at each of the electrodes is measured, and the response for each sequence is compared to that obtained from the wild-type (i.e. fully base-paired) sequences. Electrodes with attenuated signals correspond to mutated subsequences, while those which exhibited the expected charge are classified as unmutated.

Example 12: Detection of Mutations Using Electrocatalytic Currents Generated at DNA-Modified Surfaces.

The signals corresponding to mismatched and fully-paired sequences can be more highly differentiated by monitoring catalytic currents at DNA-modified surfaces. Electrons can be shuttled through the immobilized duplexes to redox-active intercalators localized on the solvent-exposed periphery of the monolayer, and then

negatively-charged solution-borne species (which are electrostatically prohibited from the interior of the monolayer) are catalytically reduced by the intercalating mediators. Since the catalytic reaction essentially amplifies the signal corresponding to the intercalator, the attenuation of this response in the presence of the mismatch is significantly more pronounced. In a specific embodiment, the sequence SH-$^{5'}$AGTACAGTCATCGCG was deposited on an electrode both hybridized with a fully base-paired complement, and with a complement containing a CA mismatch (the position of the mismatch is denoted in bold). These duplexes were immersed in a solution containing 1.0 μM methylene blue and 1 mM ferricyanide. In the presence of either of these reagents alone, only small direct currents were measured. However, in the presence of a mixture of the intercalator and the negatively charged probe, pronounced currents were measured corresponding to the electrocatalytic reduction of ferricyanide by methylene blue. The amount of current observed for the TA and CA containing films differ dramatically; using electrocatalysis, the mismatched duplex can be differentiated from the fully base-paired duplexes by a factor of approximately 100. Moreover, as illustrated in Figure 8, the peak potentials for the TA and CA duplexes are significantly separated, allowing the presence of the mismatch to be detected potentiometrically. This approach therefore represents an extremely sensitive means to detect genetic mutations electrochemically.

Example 13: Increased Sampling Time Using Electrocatalytic Currents Generated at DNA-Modified Surfaces Increases Sensitivity.

Because the charge transport-based assay features a catalytic reaction whose rate depends on the degree of complementarity within the individual duplexes, the measured charge resulting from the reduction of methylene blue at TA versus CA-containing films increases disproportionately with longer integration times (Figure 9). Using 0.5 μM methylene blue and 2.0 mM ferricyanide, 10-second potential steps to -350 mV gave faradaic charges of 36 and 6 μC respectively. Increased sampling times continue to increase the differentiation of signals obtained with mismatched versus paired complements. These results highlight the versatility of electrochemical

detection methods which are also more amenable to the portability required for a practical device.

Example 14: Detection of Genetic Mutations Within a Specific Region of the p53 Gene Using Electrocatalytic Current Measurement of Thiol-Modified Duplexes on Gold Surfaces .

Another specific embodiment involves detecting the mutations within the p53 gene using electrocatalysis. A different 20-base pair duplex derivatized with a hexylthiol linker is attached to each of approximately thirty addressable sites of a gold-microelectrode array by deposition form a concentrated duplex solution overnight. The sequences are chosen to correspond to the 600-base pair region within exons 5 through 8 of the p53 gene where most of the cancer-related mutations are found. The array is immersed in aqueous solution at 90 °C for 60 seconds to denature the immobilized duplexes and remove the complementary strands. The human sample containing the p53 gene is fragmented either before or after amplification. A solution containing the fragmented genomic single-stranded DNA is deposited on the array for one hour to allow hybridization to occur. The array is rinsed and submerged in a solution containing 1.0 μM methylene blue and 1.0 mM ferricyanide. The pronounced currents that are observed result from the electrocatalytic reduction of the solution-borne ferricyanide by methylene blue adsorbed at the solvent-exposed duplex sites. These catalytic currents are measured for each addressable electrode and compared with those obtained with the wild-type sequence to detect potential sites of mutations.

Example 15: Detection of Genetic Mutations Within a Gene of Interest Using Direct or Electrocatalytic Current Measurement of Amine-Modified Duplexes on Carbon Surfaces .

Another embodiment utilizes a carbon electrode. The electrode is oxidized at +1.5 V (vs. Ag/AgCl) in the presence of $K_2Cr_2O_7$ and HNO_3, and treated with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS). Duplexes corresponding to mutated sequences of a specific gene of interest are derivatized with a hexylamine linker and applied to the electrode surface. The device is immersed in aqueous solution at 90 °C for 60 seconds

to, generate a single-stranded monolayer, and the fragmented genomic DNA sample is hybridized to the immobilized probes at room temperature for 1 hour. The detection of mutations is accomplished by (i) measuring direct currents in the presence of 1.0 μM daunomycin solution, or (ii) by measuring catalytic currents in the presence of 1.0 μM methylene blue and 1.0 mM ferricyanide. Charges passed at each electrode are measured, and the response for each sequence is compared to that obtained with wild-type, i.e. fully base-paired, sequences. Attenuated signals correspond to mutated subsequences, while those which exhibit no change in current are classified unmutated.

Although the invention has been described with reference to particular applications, the principles involved may be used in other applications which will be apparent to those skilled in the art. The invention is accordingly to be limited only by the scope of the claims which follow.

Table I. Electrochemical Detection of Single-Base Mismatches[a]

	$Q_{c(int)}$ (nC)[b]	T_m (°C)[c]
SH-5'AGTACAGTCATCGCG TCATGTCAGTAGCGC	165(37)	68
SH-5'AGTACAGTCATCGCG TCATGTCAGCAGCGC	56(15)	56
SH-5'AGTACAGTCATCGCG TCATGTCTGTAGCGC	95(18)	57
SH-5'AGTACAGTCATCGCG TCATGTCACTAGCGC	51(23)	56
SH-5'AGTACAGTCATCGCG TCATGTCGGTAGCGC	49(30)	62
SH-5'AGTACAGTCATCGCG TCATGTAAGTAGCGC	153(38)	60
SH-5'AGTACAGTCATCGCG TCATGTCCGTAGCGC	93(17)	58

[a]Based on cyclic voltammograms measured for 1.0 μM daunomycin noncovalently bound to duplex-modified electrodes (0.1 M phosphate buffer, pH 7). Values are based on > 5 trials each, and results were comparable for experiments run side-by side, or from different sample preparation as long as electrodes exhibited high surface coverages. Electrodes with lower surface coverages yielded higher charges (> 1 DM/duplex), and decreased attenuations in the presence of mismatches. [b]Integrated background-subtracted cathodic charge. [c]Measured by monitoring duplex hypochromicity at 260 nm. Samples contained 10 μM duplex, 100 mM $MgCl_2$, 100 mM phosphate, pH 7.

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WHAT IS CLAIMED IS:

1. A composition comprising a first single stranded nucleic acid hybridized to a second single stranded nucleic acid forming a complex, said complex containing one or more electron donor moieties and one or more electron acceptor moieties combined therewith, wherein one of the electron donor or acceptor moieties is an electrode or an addressable multielectrode array and the other electron donor or acceptor moiety is an intercalative, redox-active moiety.

2. A composition according to claim 1, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length, deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a single-stranded overhang of distinct sequence composition, and wherein said single-stranded overhang is 5 to 10 nucleotides in length and can be the same or different.

3. A composition according to claim 1, wherein said intercalative, redox-active moiety is either noncovalently adsorbed or crosslinked to said oligonucleotide duplex.

4. A composition according to claim 1, wherein said intercalative, redox-active moiety is an intercalator.

5. A composition according to claim 1, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines, phenothiazines, phenazines, acridines, and anthraquinones.

6. A composition according to claim 1, wherein said intercalative, redox-active moiety is daunomycin.

7. A composition according to claim 1 wherein said intercalative, redox-active moiety is part of a protein.

8. A composition according to claim 1 wherein said intercalative, redox-active moiety is mut Y.

9. A sensor comprising:

(a) an electrode or an addressable multielectrode array;

(b) a polynucleotide duplex deposited onto at least one surface of said electrode or addressable multielectrode array, wherein said polynucleotide duplex is comprised of a first single-stranded nucleic acid hybridized to a second single-stranded nucleic acid forming a complex which possibly contains one or more mismatches or one or more base-stacking perturbations; and

(c) an intercalative, redox-active moiety contacting said polynucleotide duplex.

10. A sensor according to claim 9, wherein said intercalative, redox-active moiety is either noncovalently adsorbed or crosslinked to said polynucleotide duplex.

11. A sensor according to claim 9, wherein said intercalative, redox-active moiety is an intercalator.

12. A sensor according to claim 9, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines, phenothiazines, phenazines, acridines, and anthraquinones.

13. A sensor according to claim 9, wherein said intercalative, redox-active moiety is daunomycin.

14. A sensor according to claim 9, wherein said intercalative, redox-active moiety is part of a protein.

15. A sensor according to claim 9, wherein said intercalative, redox-active moiety is mut Y.

16. A sensor according to claim 9, wherein said electrode or addressable multielectrode array is gold.

17. A sensor according to claim 9, wherein said electrode or addressable multielectrode array is carbon.

18. A sensor according to claim 9, wherein one of said nucleic acids is derivatized with a functionalized linker.

19. A sensor according to claim 18, wherein said functionalized linker is comprised of 5 to 20 σ bonds.

20. A sensor according to claim 18, wherein said functionalized linker is thiol-terminated.

21. A sensor according to claim 18, wherein said functionalized linker is amine-terminated.

22. A sensor according to claim 9, wherein said addressable multielectrode array is comprised of oligonucleotide duplexes of 5 to 10 base-pairs in length, deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a first single-stranded overhang of known sequence composition, and wherein one of said nucleic acids has a second single-stranded overhang that is complementary to said first single-stranded overhang on said addressable multielectrode array.

23. A sensor comprising:

(a) an electrode or an addressable multielectrode array comprised of gold;

(b) one or more oligonucleotide duplexes deposited onto at least one surface of said electrode or addressable multielectrode array, wherein said oligonucleotide duplexes are comprised of a first single-stranded nucleic acid hybridized to a second single-stranded nucleic acid, and wherein one of said nucleic acids is derivatized with a thiol-terminated linker comprised of 5 to 20 σ-bonds; and

(c) an intercalative moiety site-specifically adsorbed to said oligonucleotide duplexes, wherein said intercalative moiety is daunomycin, and wherein said oligonucleotide duplexes provide electrical contact between the electrode or addressable multielectrode array and daunomycin.

24. A sensor comprising:

(a) an electrode or an addressable multielectrode array comprised of carbon;

(b) one or more oligonucleotide duplexes deposited onto at least one surface of said electrode or addressable multielectrode array, wherein said oligonucleotide duplexes are comprised of a first single-stranded nucleic acid hybridized to a second single-stranded nucleic acid, and wherein one of said nucleic acids is derivatized with an amine-terminated linker comprised of 5 to 20 σ-bonds; and

(c) an intercalative moiety site-specifically adsorbed to said oligonucleotide duplexes, wherein said intercalative moiety is daunomycin, and wherein said oligonucleotide duplexes provide electrical contact between the electrode or addressable multielectrode array and daunomycin.

25. A sensor comprising:

(a) an electrode or addressable multielectrode array, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a first single-stranded overhang of known sequence composition, and wherein said first single-stranded overhang can be the same or different;

(b) one or more target oligonucleotide duplexes deposited onto said electrode or addressable multielectrode array, wherein each of said target oligonucleotide duplexes is comprised of a first single-stranded nucleic acid hybridized to a second single-stranded nucleic acid, and wherein one of said nucleic acids contains a second single-stranded overhang complementary to said first single-stranded overhang on said electrode or addressable multielectrode array; and

(c) an intercalative moiety site-specifically adsorbed to said target oligonucleotide duplexes, wherein said intercalative moiety is daunomycin, and wherein said target oligonucleotide duplexes provide electrical contact between the electrode or addressable multielectrode array and daunomycin.

26. A method of detecting one or more base-stacking perturbations in a target sequence comprising:

(a) hybridizing a first single stranded nucleic acid to a second single stranded nucleic acid to form a first complex;

(b) depositing said first complex onto an electrode or an addressable multielectrode array;

(c) adding an intercalative, redox-active moiety to said first complex to form a second complex; and

(d) measuring an electron transfer event between said electrode or addressable multielectrode array and said intercalative, redox-active moiety as an indication for the presence or absence of said base-stacking perturbations.

27. A method according to claim 26, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

28. A method according to claim 26, wherein said intercalative, redox-active moiety is either noncovalently adsorbed or crosslinked to said first complex.

29. A method according to claim 26, wherein said intercalative, redox-active moiety is an intercalator.

30. A method according to claim 26, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines, phenothiazines, phenazines, acridines, and anthraquinones.

31. A method according to claim 26, wherein said intercalative, redox-active moiety is daunomycin.

32. A method according to claim 26, wherein said intercalative, redox-active moiety is part of a protein.

33. A method according to claim 26, wherein said intercalative, redox-active moiety is mut Y.

34. A method according to claim 26, wherein said electrode or addressable multielectrode array is gold.

35. A method according to claim 26, wherein said electrode or addressable multielectrode array is carbon.

36. A method according to claim 26, wherein one of said single-stranded nucleic acids is derivatized with a functionalized linker.

37. A method according to claim 36, wherein said functionalized linker is comprised of 5 to 20 σ bonds.

38. A method according to claim 36, wherein said functionalized linker is thiol-terminated.

39. A method according to claim 36, wherein said functionalized linker is amine-terminated.

40. A method according to claim 26, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a first single-stranded overhang of known sequence composition, and wherein one of said single-stranded nucleic acids contains a second single-stranded overhang complementary to said first single-stranded overhang on said electrode or addressable multielectrode array.

41. A method of detecting one or more base-stacking perturbations in a target sequence comprising:

(a) hybridizing a first single stranded nucleic acid to a second single stranded nucleic acid to form a first complex, wherein said nucleic acids are comprised of 12 to 25 nucleotides, and wherein one of said single-stranded nucleic acids is derivatized with a thiol-terminated linker comprised of 5 to 20 σ bonds;

(b) depositing said first complex onto an addressable gold multielectrode array;

(c) adding daunomycin to said electrode-bound first complex to form a second complex; and

(d) measuring an electron transfer event between said addressable gold multielectrode array and daunomycin as an indication for the presence or absence of said base-stacking perturbations.

42. A method according to claim 41, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

43. A method of detecting one or more base-stacking perturbations in a target sequence comprising:

(a) hybridizing a first single stranded nucleic acid to a second single stranded nucleic acid to form a first complex, wherein said nucleic acids are comprised of 12 to 25 nucleotides, and wherein one of said single-stranded nucleic acids is derivatized with a amine-terminated linker comprised of 5 to 20 σ bonds;

(b) depositing said first complex onto an addressable carbon multielectrode array;

(c) adding daunomycin to said electrode-bound first complex to form a second complex; and

(d) measuring an electron transfer event between said addressable carbon multielectrode array and daunomycin as an indication for the presence or absence of said base-stacking perturbations.

44. A method according to claim 43, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

45. A method of detecting one or more base-stacking perturbations in a target sequence comprising:

(a) hybridizing a first single-stranded nucleic acid to a second single-stranded nucleic acid to form a first complex of 12 to 25 nucleotides in length,

wherein said first complex contains a first single-stranded overhang of known sequence composition, and wherein said first single-stranded overhang can be the same or different;

(b) depositing said first complex onto an addressable multielectrode array, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a second single-stranded overhang complementary to said first single-stranded overhang;

(c) adding daunomycin to said electrode-bound first complex to form a second complex; and

(d) measuring an electron transfer event between said addressable multielectrode array and daunomycin as an indication for the presence or absence of said base-stacking perturbations.

46. A method according to claim 45, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

47. A method of continuously detecting one or more base-stacking perturbations comprising:

(a) hybridizing a single stranded nucleic acid derivatized with a functionalized linker to a first target sequence to form a first complex;

(b) depositing said first complex onto an electrode or an addressable multielectrode array;

(c) adsorbing an intercalative, redox-active moiety to said electrode-bound first complex to form a second complex;

(d) measuring an electron transfer event between said electrode or addressable multielectrode array and said intercalative, redox-active moiety in said

second complex as an indication for the presence or absence of said base-stacking perturbations;

(e) disrupting said second complex by applying denaturing conditions, removing said first target sequence and said intercalative, redox-active moiety, and generating a single-stranded monolayer;

(f) adding a second target sequence to said single-stranded monolayer under hybridizing conditions to form a third complex;

(g) adsorbing an intercalative, redox-active moiety to said third complex to form a fourth complex;

(h) measuring an electron transfer event between said electrode or addressable multielectrode array and said intercalative, redox-active moiety in said fourth complex as an indication for the presence or absence of said base-stacking perturbations; and

(i) repeating steps (e) through (h) using multiple target sequences.

48. A method according to claim 47, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

49. A method according to claim 47, wherein said intercalative, redox-active moiety is an intercalator.

50. A method according to claim 47, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines, phenothiazines, phenazines, acridines, and anthraquinones.

51. A method according to claim 47, wherein said intercalative, redox-active moiety is daunomycin.

52. A method according to claim 47, wherein said intercalative, redox-active moiety is part of a protein.

53. A method according to claim 47, wherein said intercalative, redox-active moiety is mut Y.

54. A method according to claim 47, wherein said electrode or addressable multielectrode array is gold.

55. A method according to claim 47, wherein said electrode or addressable multielectrode array is carbon.

56. A method according to claim 47, wherein said functionalized linker is comprised of 5 to 20 σ bonds.

57. A method according to claim 47, wherein said functionalized linker is thiol-terminated.

58. A method according to claim 47, wherein said functionalized linker is amine-terminated.

59. A method of continuously detecting one or more base-stacking perturbations comprising:

(a) hybridizing a first single stranded nucleic acid to a second single-stranded nucleic acid to form a first target duplex, wherein said first target duplex contains a single-stranded overhang of known sequence composition, and wherein said first single-stranded overhang can be the same or different;

(b) depositing said first target duplex onto an electrode or addressable multielectrode array to form a first complex, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a second single-stranded overhang complementary to said first single-stranded overhang;

(c) adsorbing an intercalative, redox-active moiety to said first complex to form a second complex;

(d) measuring an electron transfer event between said electrode or addressable multielectrode array and said intercalative, redox-active moiety in said

second complex as an indication for the presence or absence of said base-stacking perturbations;

(e) disrupting said second complex by applying denaturing conditions and removing said first target duplex and said intercalative, redox-active moiety;

(f) adding a second target duplex containing a single-stranded overhang under hybridizing conditions to form a third complex;

(g) adsorbing an intercalative, redox-active moiety to said third complex to form a fourth complex;

(h) measuring an electron transfer event between said electrode or addressable multielectrode array and said intercalative, redox-active moiety in said fourth complex as an indication for the presence or absence of said base-stacking perturbations; and

(i) repeating steps (e) through (h) using multiple target duplexes.

60. A method according to claim 59, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

61. A method according to claim 59, wherein said intercalative, redox-active moiety is an intercalator.

62. A method according to claim 59, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines, phenothiazines, phenazines, acridines, and anthraquinones.

63. A method according to claim 59, wherein said intercalative, redox-active moiety is daunomycin.

64. A method according to claim 59, wherein said intercalative, redox-active moiety is part of a protein.

65. A method according to claim 59, wherein said intercalative, redox-active moiety is mut Y.

66. A method of continuously detecting one or more base-stacking perturbations comprising:

(a) hybridizing a single stranded nucleic acid which is derivatized with a thiol-terminated linker comprised of 5 to 20 σ bonds to a first nucleic acid target sequence to form a first complex, wherein said nucleic acids are comprised of 12 to 25 nucleotides

(b) depositing said first complex onto an addressable gold multielectrode array;

(c) adsorbing daunomycin to said first complex to form a second complex;

(d) measuring an electron transfer event between said gold multielectrode array and daunomycin in said second complex as an indication for the presence or absence of said base-stacking perturbations;

(e) disrupting said second complex by immersing it in aqueous solution at elevated temperatures for 1 minute, removing said first target sequence and daunomycin, and generating a single-stranded monolayer;

(f) adding a second target sequence to said single-stranded monolayer under hybridizing conditions to form a third complex;

(g) adsorbing daunomycin to said third complex to form a fourth complex;

(h) measuring an electron transfer event between said gold multielectrode array and daunomycin in said fourth complex as an indication for the presence or absence of said base-stacking perturbations; and

(i) repeating steps (e) through (h) using multiple target sequences.

67. A method according to claim 66, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

68. A method of continuously detecting one or more base-stacking perturbations comprising:

(a) hybridizing a single stranded nucleic acid which is derivatized with an amine-terminated linker comprised of 5 to 20 σ bonds to a first nucleic acid target sequence to form a first complex, wherein said nucleic acids are comprised of 12 to 25 nucleotides

(b) depositing said first complex onto an addressable carbon multielectrode array;

(c) adsorbing daunomycin to said first complex to form a second complex;

(d) measuring an electron transfer event between said carbon multielectrode array and daunomycin in said second complex as an indication for the presence or absence of said base-stacking perturbations;

(e) disrupting said second complex by immersing it in aqueous solution at elevated temperatures for 1 minute, removing said first target sequence and daunomycin, and generating a single-stranded monolayer;

(f) adding a second target sequence to said single-stranded monolayer under hybridizing conditions to form a third complex;

(g) adsorbing daunomycin to said third complex to form a fourth complex;

(h) measuring an electron transfer event between said carbon multielectrode array and daunomycin in said fourth complex as an indication for the presence or absence of said base-stacking perturbations; and

(i) repeating steps (e) through (h) using multiple target sequences.

69. A method according to claim 68, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

70. A method of continuously detecting one or more base-stacking perturbations comprising:

(a) hybridizing a first single stranded nucleic acid to a second single-stranded nucleic acid to form a first target duplex of 12 to 25 nucleotides in length, wherein said first target duplex contains a single-stranded overhang of known sequence composition, and wherein said first single-stranded overhang can be the same or different;

(b) depositing said first target duplex onto an addressable multielectrode array to form a first complex, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a second single-stranded overhang complementary to said first single-stranded overhang;

(c) adsorbing daunomycin to said first complex to form a second complex;

(d) measuring an electron transfer event between said addressable multielectrode array and daunomycin in said second complex as an indication for the presence or absence of said base-stacking perturbations;

(e) disrupting said second complex by immersing it in aqueous solution at elevated temperatures for 1 minute, removing said first target duplex and daunomycin, and regenerating said monolayer of oligonucleotide duplexes;

(f) adding a second target duplex to said monolayer of oligonucleotide duplexes under hybridizing conditions, forming a third complex;

(g) adsorbing daunomycin to said third complex to form a fourth complex;

(h) measuring an electron transfer event between said addressable multielectrode array and daunomycin in said fourth complex as an indication for the presence or absence of said base-stacking perturbations; and

(i) repeating steps (e) through (h) using multiple target duplexes.

71. A method according to claim 70, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

72. A method of detecting one or more point mutations within the p53 gene, comprising:

(a) forming a set of oligonucleotide duplexes of approximately 20 base-pairs in length corresponding to the approximately 600 base pair long region within exons 5 through 8 of the p53 gene, wherein said oligonucleotide duplexes are derivatized with a thiol-terminated linker comprised of 5 to 20 σ bonds;

(b) depositing said oligonucleotide duplexes onto an addressable gold multielectrode array;

(c) denaturing said oligonucleotide duplexes by immersing them in aqueous solution at elevated temperatures for 1 minute and removing the complementary strands to form a single-stranded monolayer;

(d) exposing said single-stranded monolayer to a first sample comprising PCR-amplified and fragmented p53 gene DNA under hybridizing conditions to form a first complex;

(e) rinsing said electrode-bound first complex to remove any unhybridized material;

(f) adsorbing daunomycin to said first complex by immersing it in a dilute solution of daunomycin to form a second complex;

(g) measuring an electron transfer event between said gold multielectrode array and daunomycin in said second complex as an indication for the presence or absence of said point mutations;

(h) denaturing said second complex by immersing it in an aqueous solution at elevated temperatures for 1 minute, removing daunomycin and the

complementary strands from said first sample, and regenerating said single-stranded monolayer;

(i) exposing said single-stranded monolayer to a second sample containing PCR-amplified and fragmented p53 gene DNA under hybridizing conditions to form a third complex; and

(k) repeating steps (e) through (h) using several sample solutions.

73. A method of detecting one or more point mutations within the p53 gene, comprising:

(a) generating a first sample set of oligonucleotide duplexes of approximately 20 base-pairs in length corresponding to the approximately 600 base pair long region within exons 5 through 8 of the p53 gene, wherein said oligonucleotide duplexes contain PCR-amplified and fragmented p53 gene DNA, and wherein said oligonucleotide duplexes contain a first single-stranded overhang of known composition;

(b) depositing said oligonucleotide duplexes onto an addressable multielectrode array to form a first complex, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a second single-stranded overhang complementary to said first single-stranded overhang;

(c) adsorbing daunomycin to said first complex by immersing it in a dilute solution of daunomycin to form a second complex;

(d) measuring an electron transfer event between said multielectrode array and daunomycin in said second complex as an indication for the presence or absence of said point mutations;

(e) disrupting said second complex by immersing it in aqueous solution at elevated temperatures for 1 minute, removing said first sample set of

oligonucleotide duplexes, and regenerating said monolayer of oligonucleotide duplexes;

(f) exposing said monolayer to a second sample set of oligonucleotide duplexes to form a third complex, wherein said oligonucleotide duplexes contain PCR-amplified and fragmented p53 gene DNA, and wherein said oligonucleotide duplexes contain a third single-stranded overhang complementary to said second single-stranded overhang; and

(h) repeating steps (c) through (f) using several sample sets.

74. A method of detecting one or more base-stacking perturbations electrocatalytically in a target sequence comprising:

(a) hybridizing a first single stranded nucleic acid to a second single stranded nucleic acid to form a first complex;

(c) depositing said first complex onto an electrode or an addressable multielectrode array to form a second complex;

(d) immersing said second complex in a solution comprising an intercalative, redox-active species and a non-intercalative, redox-active species; and

(d) measuring an electron transfer event as an indication for the presence or absence of said base-stacking perturbations.

75. A method according to claim 74, wherein said base-stacking perturbations are point mutations, protein-DNA adducts, adducts between any chemical entity and said target sequence, or combinations thereof.

76. A method according to claim 74, wherein said intercalative, redox-active moiety is an intercalator.

77. A method according to claim 74, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines, phenothiazines, phenazines, acridines, and anthraquinones.

78. A method according to claim 74, wherein said non-intercalative, redox-active moiety is selected from the group consisting of ferricyanide, ferrocenes, hexacyanoruthenate, and hexacyanoosmate.

79. A method according to claim 74, wherein said intercalative, redox-active moiety is a protein.

80. A method according to claim 74, wherein said intercalative, redox-active moiety is methylene blue, and wherein said non-intercalative, redox-active moiety is ferricyanide.

81. A method according to claim 74, wherein said electrode or addressable multielectrode array is gold.

82. A method according to claim 74, wherein said electrode or addressable multielectrode array is carbon.

83. A method according to claim 74, wherein one of said single-stranded nucleic acids is derivatized with a functionalized linker.

84. A method according to claim 83, wherein said functionalized linker is comprised of 5 to 20 σ bonds.

85. A method according to claim 83, wherein said functionalized linker is thiol-terminated.

86. A method according to claim 83, wherein said functionalized linker is amine-terminated.

87. A method according to claim 74, wherein said addressable multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes are derivatized on one end with a functionalized linker and on the opposite end with a first single-stranded overhang of distinct sequence composition, and wherein one of said single-stranded nucleic acids contains a second single-stranded overhang complementary to said first single-stranded overhang on said electrode or addressable multielectrode array.

88. A method of detecting one or more point mutations electrocatalytically within the p53 gene, comprising:

(a) forming a set of oligonucleotide duplexes of approximately 20 base-pairs in length corresponding to the approximately 600 base pair long region within exons 5 through 8 of the p53 gene, wherein said oligonucleotide duplexes are derivatized with a thiol-terminated linker comprised of 5 to 20 σ bonds;

(b) depositing said oligonucleotide duplexes onto an addressable gold multielectrode array;

(c) denaturing said oligonucleotide duplexes by immersing them in aqueous solution at elevated temperatures for 1 minute and removing the complementary strands to form a single-stranded monolayer;

(d) exposing said single-stranded monolayer to a first sample comprising PCR-amplified and fragmented p53 gene DNA under hybridizing conditions to form a first complex;

(e) rinsing said electrode-bound first complex to remove any unhybridized material;

(f) immersing said electrode-bound first complex into a dilute solution comprised of 1.0 μM methylene blue and 1.0 mM ferricyanide;

(g) measuring an electron transfer event as an indication for the presence or absence of said point mutations;

(h) denaturing said electrode-bound first complex by immersing it in an aqueous solution at elevated temperatures for 1 minute, and regenerating said single-stranded monolayer;

(i) exposing said single-stranded monolayer to a second sample containing PCR-amplified and fragmented p53 gene DNA under hybridizing conditions to form a second complex; and

(k) repeating steps (e) through (h) using several sample solutions.

89. A method of detecting one or more point mutations electrocatalytically within the p53 gene, comprising:

(a) generating a first sample set of oligonucleotide duplexes of approximately 20 base-pairs in length corresponding to the approximately 600 base pair long region within exons 5 through 8 of the p53 gene, wherein said oligonucleotide duplexes contain PCR-amplified and fragmented p53 gene DNA, and wherein said oligonucleotide duplexes contain a first single-stranded overhang of known composition;

(b) depositing said oligonucleotide duplexes onto an addressable gold multielectrode array to form a first complex, wherein said addressable gold multielectrode array is comprised of a monolayer of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a second single-stranded overhang complementary to said first single-stranded overhang;

(c) immersing said electrode-bound first complex into a dilute solution comprised of 1.0 μM methylene blue and 1.0 mM ferricyanide;

(d) measuring an electron transfer event as an indication for the presence or absence of said point mutations;

(e) disrupting said second complex by immersing it in aqueous solution at elevated temperatures for 1 minute, removing said first sample set of oligonucleotide duplexes, and regenerating said monolayer of oligonucleotide duplexes;

(f) exposing said monolayer to a second sample set of oligonucleotide duplexes to form a third complex, wherein said oligonucleotide duplexes contain PCR-amplified and fragmented p53 gene DNA, and wherein said oligonucleotide duplexes contain a third single-stranded overhang complementary to said second single-stranded overhang; and

(h) repeating steps (c) through (f) using several sample sets.

90. A method of detecting the presence of a protein in a solution comprising:

(a) hybridizing a first single stranded nucleic acid to a second single stranded nucleic acid to form a first complex, wherein the sequence composition of said first complex comprises the recognition sequence of said protein;

(b) depositing said first complex onto an electrode or an addressable multielectrode array;

(c) adding an intercalative, redox-active moiety to said first complex to form a second complex;

(d) immersing said second complex into a first sample solution which potentially comprises said protein;

(e) measuring an electron transfer event between said electrode or addressable multielectrode array and said intercalative, redox-active moiety of said second complex as an indication for the presence or absence of said protein in said first sample solution;

(f) rinsing said second complex;

(g) immersing said second complex into a second sample solution which potentially comprises said protein; and

(h) repeating steps (e) through (g) using multiple sample solutions.

91. A method according to claim 90, wherein said intercalative, redox-active moiety is either noncovalently adsorbed or crosslinked to said first complex.

92. A method according to claim 90, wherein said intercalative, redox-active moiety is an intercalator.

93. A method according to claim 90, wherein said intercalative, redox-active moiety is an intercalator selected from the group consisting of phenanthridines, phenothiazines, phenazines, acridines, and anthraquinones.

94. A method according to claim 90, wherein said intercalative, redox-active moiety is daunomycin.

95. A method according to claim 90, wherein said electrode or addressable multielectrode array is gold.

96. A method according to claim 90, wherein said electrode or addressable multielectrode array is carbon.

97. A method according to claim 90, wherein one of said nucleic acids is derivatized with a functionalized linker.

98. A method according to claim 97, wherein said functionalized linker is comprised of 5 to 20 σ bonds.

99. A method according to claim 97, wherein said functionalized linker is thiol-terminated.

100. A method according to claim 97, wherein said functionalized linker is amine-terminated.

101. A method according to claim 90, wherein said addressable multielectrode array is comprised of oligonucleotide duplexes of 5 to 10 base-pairs in length deposited onto said array, wherein each of said oligonucleotide duplexes is derivatized on one end with a functionalized linker and on the opposite end with a first single-stranded overhang of distinct sequence composition, and wherein one of said nucleic acids has a second single-stranded overhang that is complementary to said first single-stranded overhang on said addressable multielectrode array.

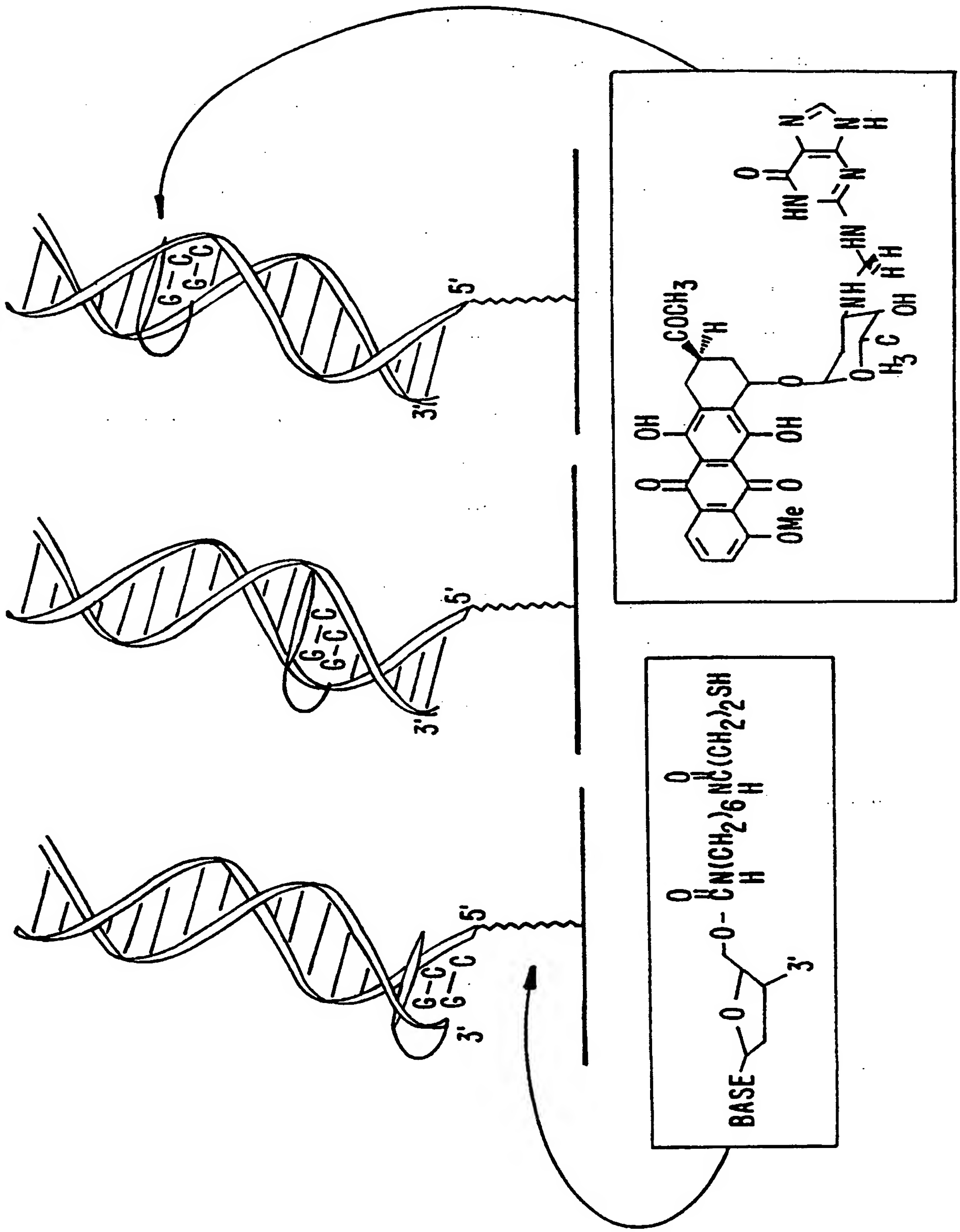
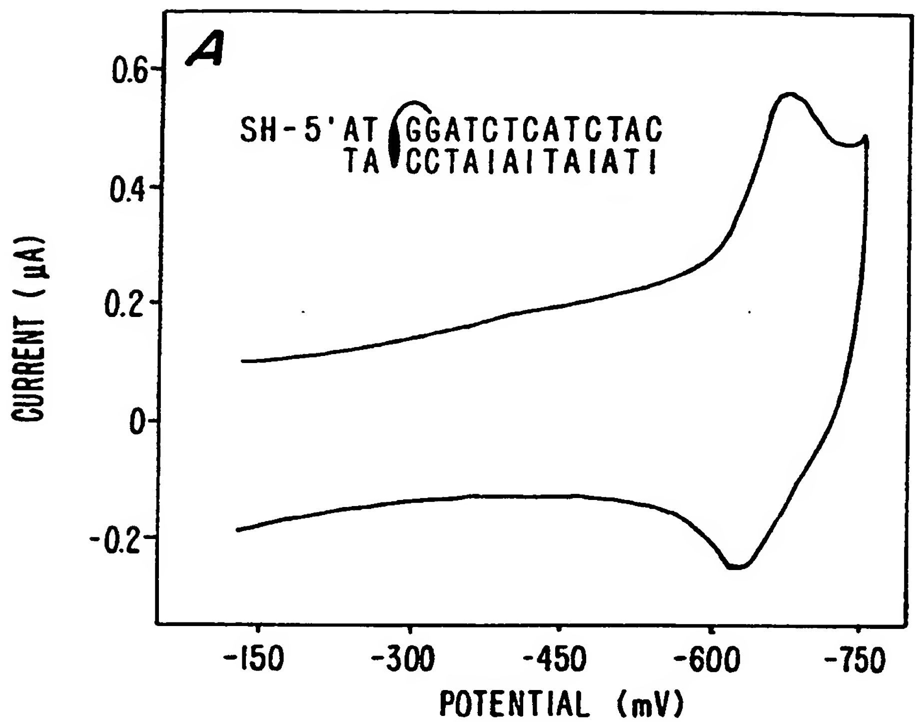


FIG. 1.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TACCTAIAITAIATI
0.6
0.4
0.2
0
-0.2
CURRENT (μA)
-150
-300
-450
-600
-750
POTENTIAL (mV)

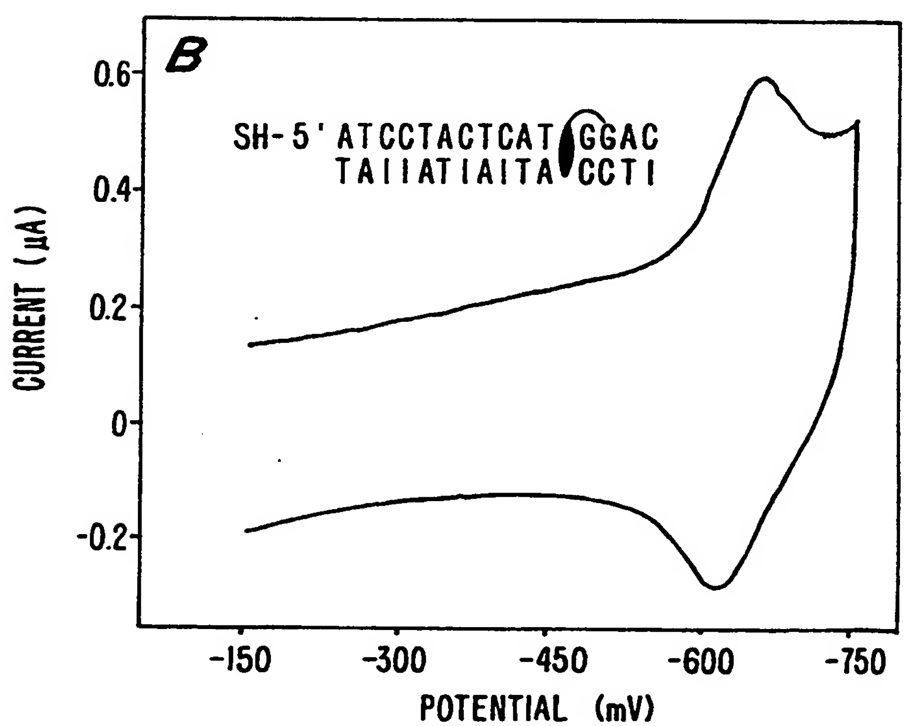

B
SH-5'ATCCTACTCATGGAC
TAIIATIAITACCTI
0.6
0.4
0.2
0
-0.2
CURRENT (μA)
-150
-300
-450
-600
-750
POTENTIAL (mV)

FIG. 2.

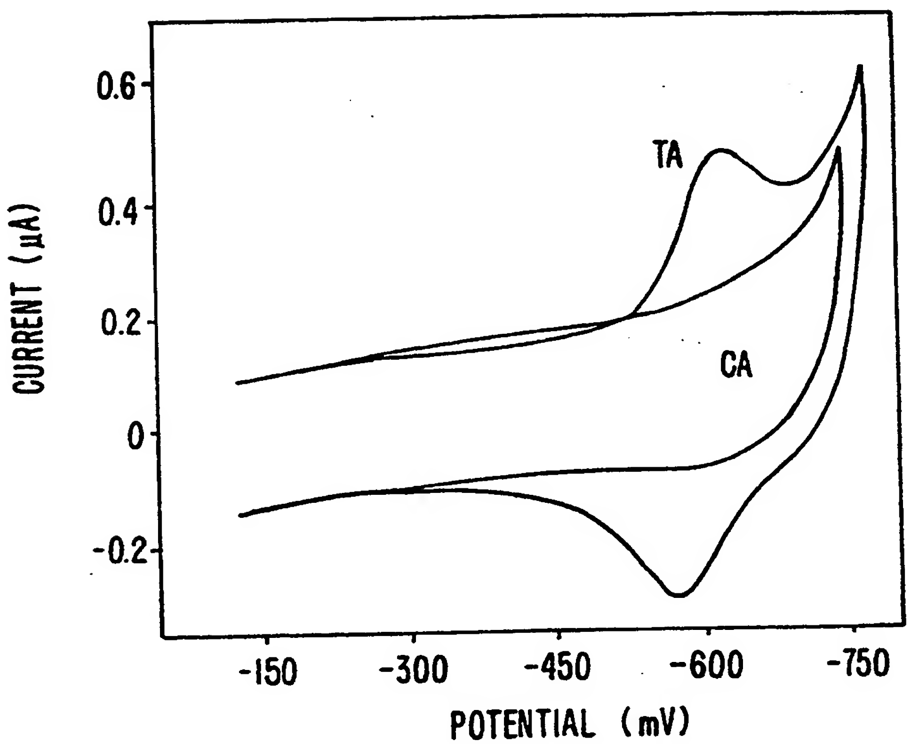


FIG. 3.

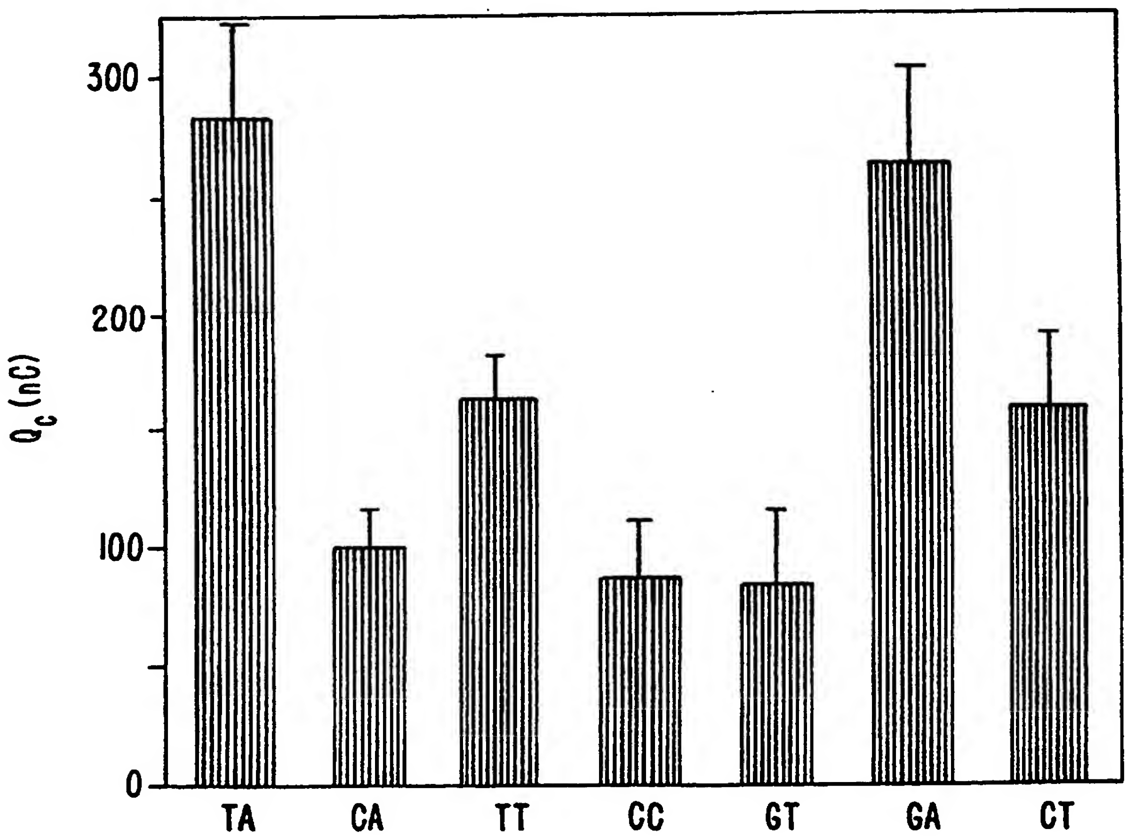


FIG. 4.

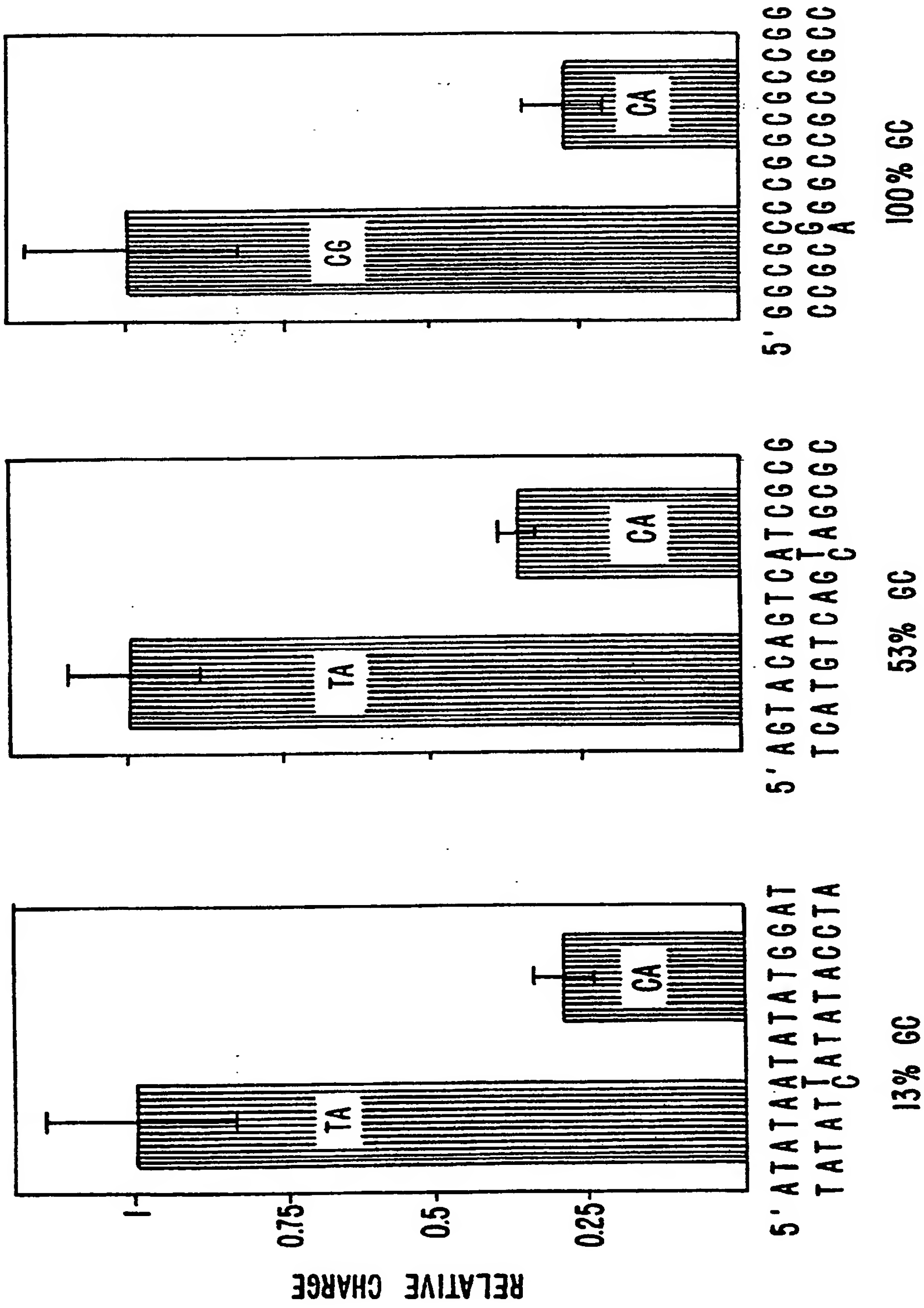


FIG. 5.

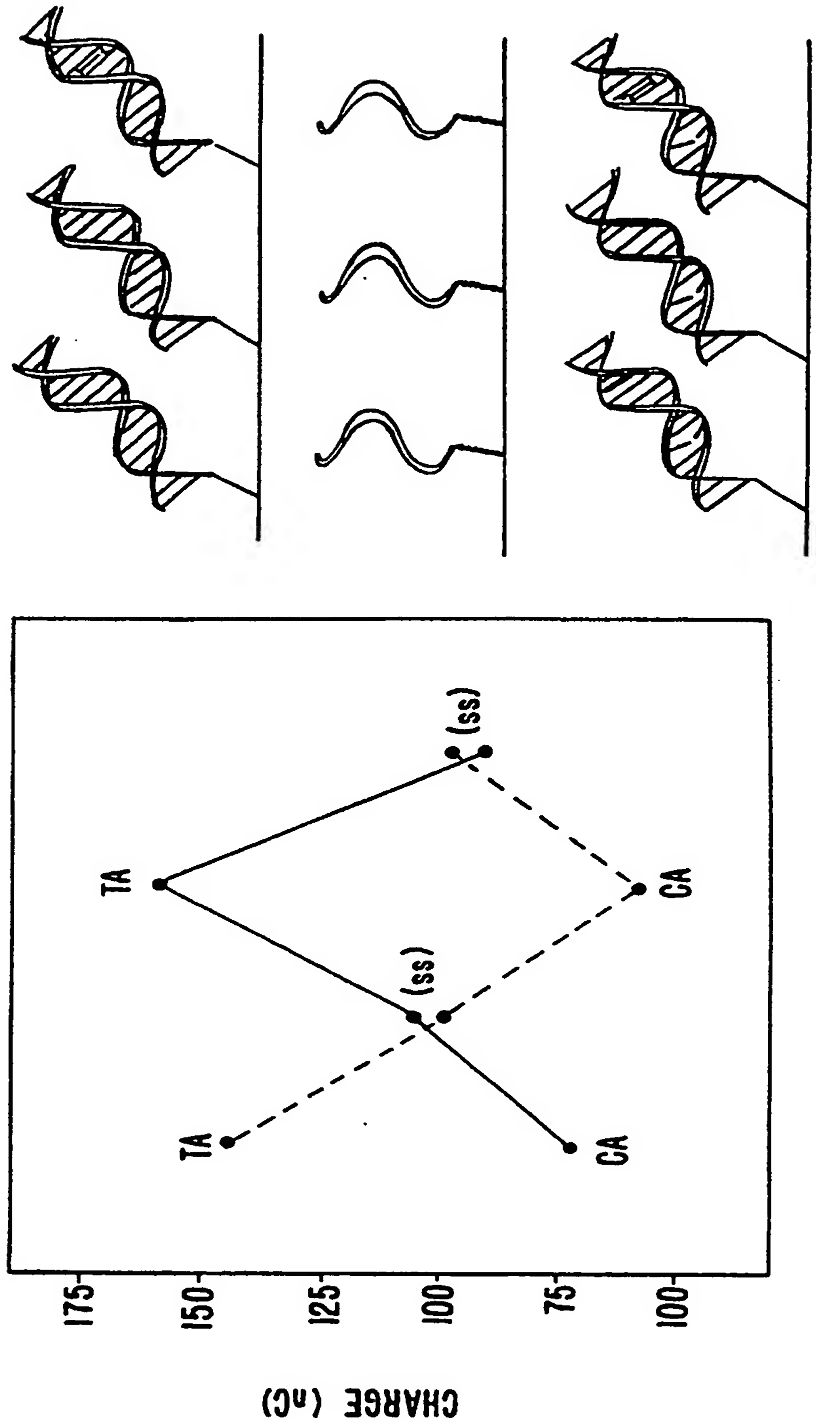


FIG. 6.

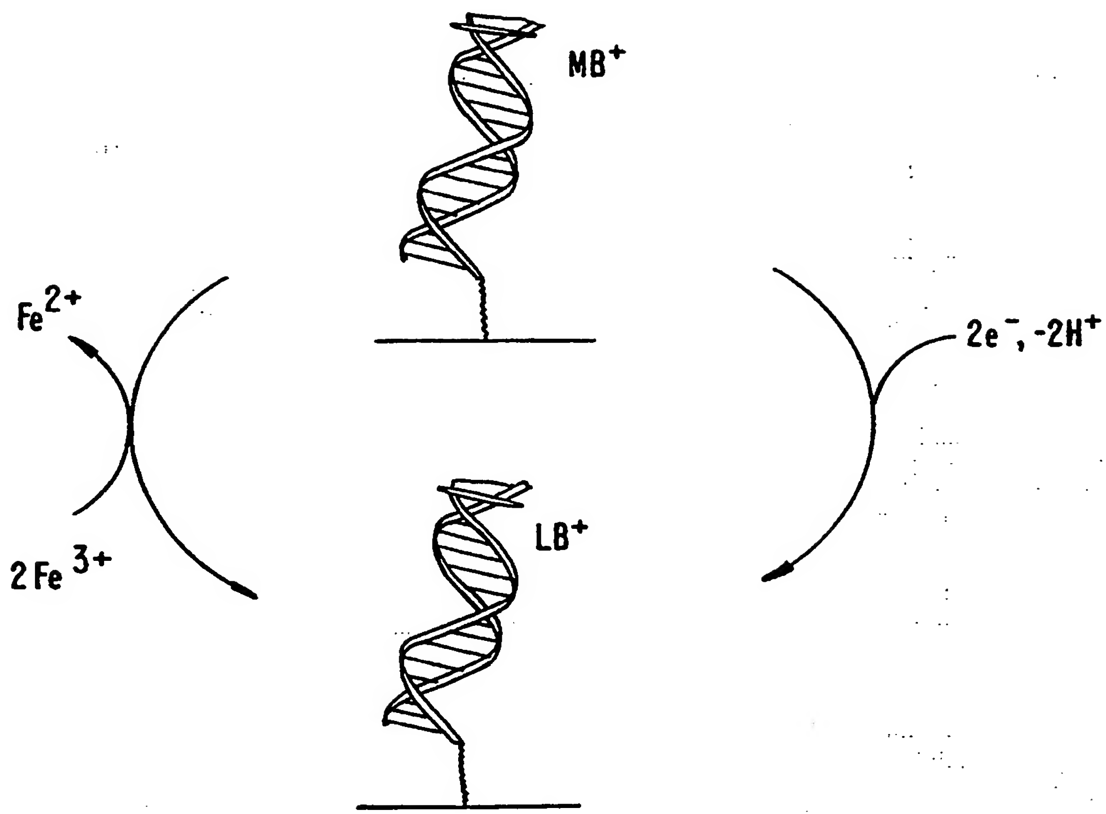
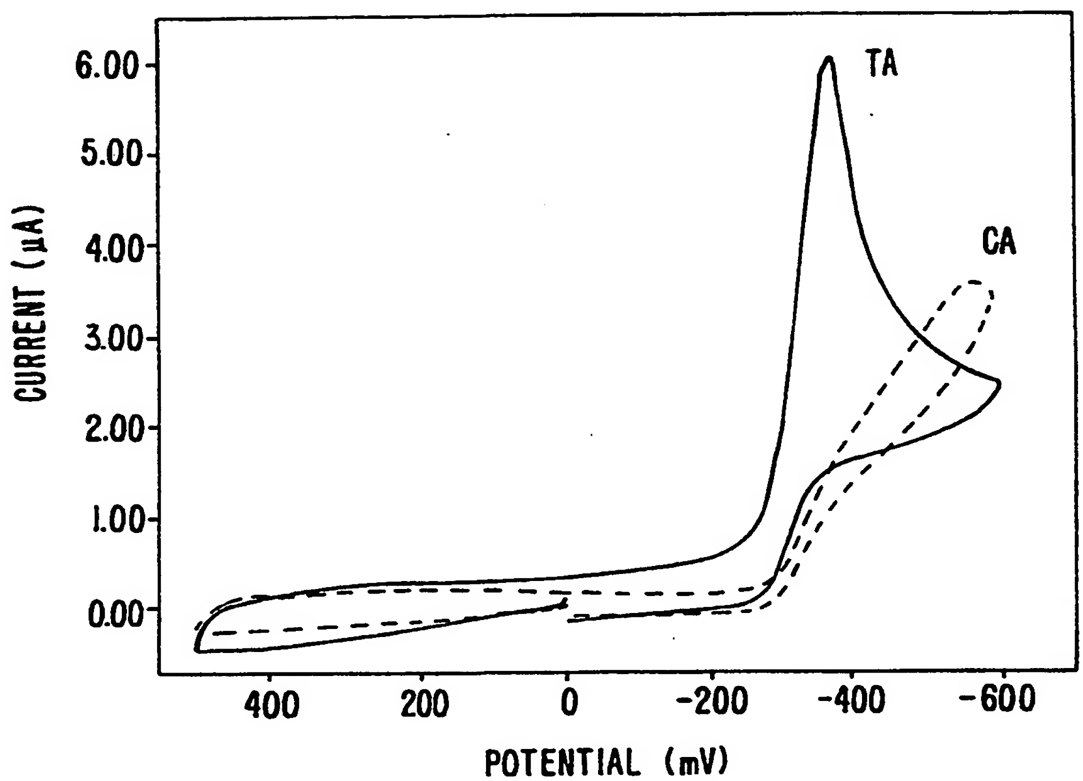


FIG. 7.



6.00
5.00
4.00
3.00
2.00
1.00
0.00
CURRENT (μA)
TA
CA
400
200
0
-200
-400
-600
POTENTIAL (mV)

FIG. 8.

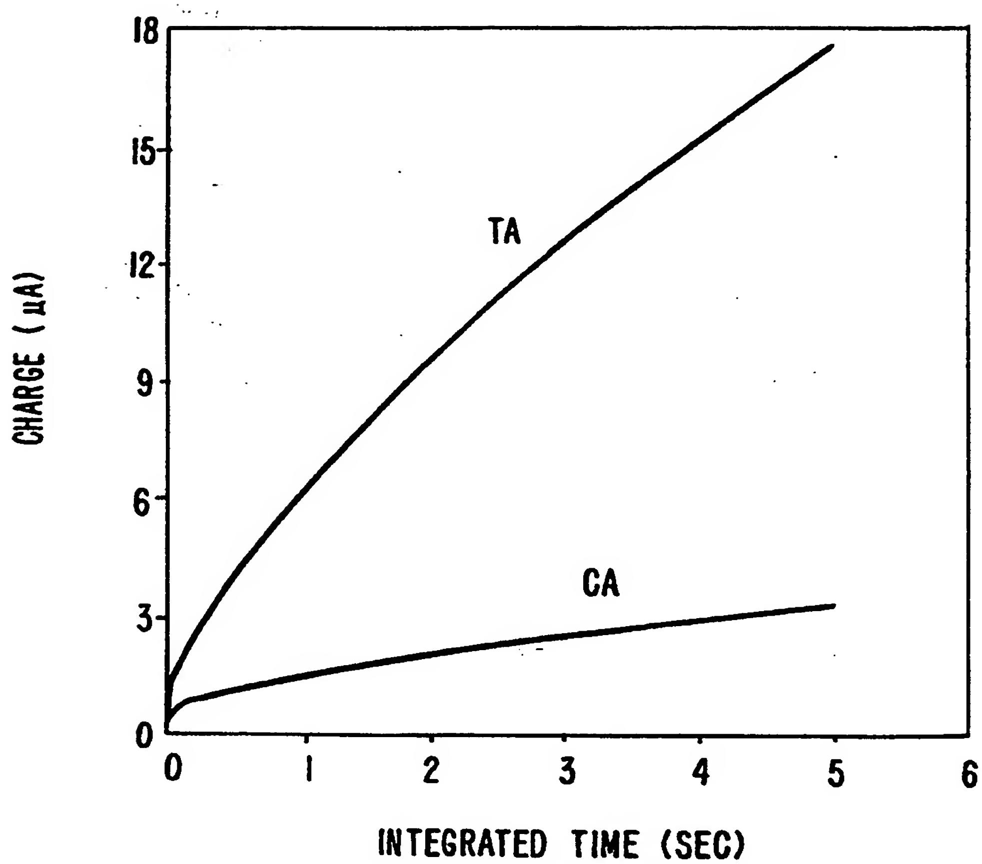


FIG. 9.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/07650

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N27/327 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 15971 A (CALIFORNIA INSTITUTE OF TECHNOLOGY) 15 June 1995 (1995-06-15) abstract; claims ---	1,3,26, 28,41, 43,45, 47,59, 66,68, 70,74
A	US 5 312 527 A (MIKKELSEN ET AL.) 17 May 1994 (1994-05-17) column 4 - column 6; figure 3 --- -/--	1,9, 22-26, 41,43, 45,47, 59,66, 68,70,74

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 July 1999	03. 09. 1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mosser

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/07650

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HASHIMOTO, K. ET AL.: "Novel DNA sensor for electro chemical gene detection" ANALYTICA CHIMICA ACTA, vol. 286, 1994, pages 219-224, XP002900561 the whole document ---	1-71, 74-87
A	JOHNSTON, D.H. ET AL.: "Electrochemical Measurement of the Solvent Accessibility of Nucleobases Using Electron Transfer between DNA and Metal Complexes" J. CHEM. SOC., vol. 117, 1995, pages 8933-8938, XP002900562 page 8933; figures 1,2; tables 1,2 ---	1,3,9, 10,26, 28,74, 78,90,91
A	CHEMICAL ABSTRACTS, vol. 120, no. 11, 14 March 1994 (1994-03-14) Columbus, Ohio, US; abstract no. 126263c, HASHIMOTO ET AL.: "DNA sensor: a novel electrochemical gene defection method using carbon electrode immobilized DNA probes" page 246; column 2; XP002900563 abstract & SUPRAMOL. CHEM., vol. 2, no. 4, 1993, pages 265-270, ---	1,4,5, 9-12,17, 24,26, 28-30, 35,41, 43,45, 47,49, 50,55, 59,61, 62,66, 74,76, 77,82
A	CHEMICAL ABSTRACTS, vol. 127, no. 2, 14 July 1997 (1997-07-14) Columbus, Ohio, US; abstract no. 13828s, WANG ET AL.: "Detection of point mutation in the p53 gene using a peptide nucleic acid biosensor" page 135; column 1; XP002900564 abstract & ANAL. CHIM. ACTA, vol. 344, no. 1-2, 1997, pages 111-118, -----	72,73, 88,89

INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/US 99/07650

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